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(54) Title: NOVEL MOLECULES OF THE CARD-RELATED PROTEIN FAMILY AND USES THEREOF

(57) Abstract: Novel CARD-12 polypeptides, proteins, and nucleic acid molecules are disclosed. In addition to isolated CARD-12 proteins, the invention further provides CARD-12, fusion proteins, antigenic peptides and anti-CARD-12 antibodies. The invention also provides CARD-12 nucleic acid molecules, recombinant expression vectors containing a nucleic acid molecule of the invention, host cells into which the expression vectors have been introduced and non-human transgenic animals in which a CARD-12 gene has been introduced or disrupted. Diagnostic, screening and therapeutic methods utilizing compositions of the invention are also provided.

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### Related Application Information

## Background of the Invention

In differentiated cells, a particular type of cell death called apoptosis occurs when an internal suicide program is activated. This program can be initiated by a variety of external signals as well as signals that are generated within the cell in response to, for example, genetic damage. For many years, the magnitude of apoptotic cell death was not appreciated because the dying cells are quickly eliminated by phagocytes, without an inflammatory response.

Caspases, a class of proteins central to the apoptotic program, are responsible for the degradation of cellular proteins that leads to the morphological changes seen in cells undergoing apoptosis. Caspases (cysteinyl aspartate-specific proteinases) are cysteine

proteases having specificity for aspartate at the substrate cleavage site. Generally, caspases are classified as either initiator caspases or effector caspases, both of which are zymogens that are activated by proteolysis that generates an active species. An effector caspase is activated by an initiator caspase which cleaves the effector caspase. Initiator caspases are activated by an autoproteolytic mechanism that is often dependent upon  
5 oligomerization directed by association of the caspase with an adapter molecule.

Apoptotic signaling is dependent on protein-protein interactions. At least three different protein-protein interaction domains, the death domain, the death effector domain and the caspase recruitment domain (CARD), have been identified within proteins  
10 involved in apoptosis. A fourth protein-protein interaction domain, the death recruiting domain (DRD) was recently identified in murine FLASH (Imai et al. (1999) *Nature* 398:777-85).

Caspases comprise a multi-gene family having at least 12 distinct family members (Nicholson (1999) *Cell Death and Differentiation* 6:1028). A relatively small fraction of  
15 cellular polypeptides (less than 200) are thought to serve as targets for cleavage by caspases. Because many of these caspase targets perform key cellular functions, their proteolysis is thought to account for the cellular and morphological events that occur during apoptosis. Members of the caspase gene family can be divided by phylogenetic analysis into two major subfamilies, based upon their relatedness to ICE (interleukin-1 $\beta$   
20 converting enzyme; caspase-1) and CED-3. Alternate groupings of caspases can be made based upon their substrate specificities.

Many caspases and proteins that interact with caspases possess a CARD domain. Hofmann et al. ((1997) *TIBS* 22:155) and others have postulated that certain apoptotic proteins bind to each other via their CARD domains and that different subtypes of CARD  
25 domains may confer binding specificity, regulating the activity of various caspases, for example.

Apoptosis in mammalian cells is mediated by large protein families that share sequence and structural similarity with the core apoptotic proteins of *Caenorhabditis elegans* (Metzstein et al. (1998) *Trends. Genet.* 14:410). The nematode CED-4 protein  
30 and its human homolog Apaf-1 play central roles in apoptosis by transducing death signals to the activation of caspases. Both CED-4 and Apaf-1 contain an N-terminal CARD domain that mediates caspase binding and a centrally located nucleotide-binding site (NBS) domain. Unlike CED-4, Apaf-1 contains a C-terminal WD-40 domain that mediates protein activation in response to the release of mitochondrial cytochrome c (Zou  
35 et al. (1997) *Cell* 90:405; Li et al. (1997) *Cell* 91:479; Srinivasula et al. (1998) *Mol. Cell.* 1:949). Additional CED4/Apaf-1 family members include CARD-4, Nod2 and CARD-7

(NAC/DEFCAP) (Bertin et al. (1999) J. Biol. Chem. 274:12955; Bertin et al. (2000) J. Biol. Chem. 275:41082; Inohara et al. (2000) J. Biol. Chem. 275:27823; Chu et al. (2001) J. Biol. Chem. 276:9239; Hliang et al. (2001) J. Biol. Chem. 276:9230). CARD4, Nod2 and CARD7 each contain NBS domains and effector CARD domains that mediate binding to downstream CARD-containing signaling partners. Both CARD-4 and Nod2 assemble together with the CARD protein RICK and induce the activation of NF-kB. Recent evidence suggests that CARD-7 may play a role analogous to Apaf-1 and directly mediate caspase activation. In addition, each protein contains extensive leucine-rich repeats (LRR) that have been proposed to function as binding sites for upstream regulators. The structure of CARD-4, Nod2 and CARD-7 is strikingly similar to plant NBS/LRR proteins that induce gene expression and cell death in response to pathogen infection (Dixon et al. (2000) Proc. Nat'l. Acad. Sci. USA 97:8807). Thus, CARD-4, Nod2 and CARD-7 likely play critical roles in stress-activated signaling pathways and may be components of the host innate immune response.

#### Summary of the Invention

The invention features nucleic acid molecules encoding human CARD-12. CARD-12 has a CARD domain, a nucleotide binding site (NBS) domain, and a leucine rich repeat (LRR) domain. These domains are found in a number of proteins that transmit signals that activate apoptotic and inflammatory pathways in response to stress and other stimuli. Upon activation, CARD-12, like Apaf-1 (Zou et al. (1997) Cell 90:405-413), likely binds a nucleotide, allowing CARD-12 to bind to and activate a CARD-containing protein via a CARD-CARD interaction, leading to modulation of apoptosis.

CARD-12 nucleic acids and polypeptides, as well as modulators of CARD-12 activity or expression, are expected to be useful in the modulation of stress-related, apoptotic and inflammatory responses, e.g., for the treatment of apoptotic and inflammatory disorders. In addition, CARD-12 nucleic acids and polypeptides are expected to be useful in the diagnosis of apoptotic and inflammatory disorders as well as in screening assays which can be used to identify compounds which can be used to modulate stress-related, apoptotic and inflammatory responses.

Many cytoplasmic plant proteins involved in response to plant pathogens, generally referred to as "R" proteins, have both an NBS domain and an LRR domain (van der Bizen and Jones (1999) Current Biology 8:226-228). R proteins are involved in both a rapid defense response (hypersensitive response) and more long-term nonspecific resistance (systemic acquired resistance). The hypersensitive response involves cell and



tissue death that is localized to the site of infection. The LRR domains of R proteins are believed to recognize and bind to pathogen proteins, triggering defensive responses. Many R proteins have an amino terminal effector domain (e.g., a TIR domain or a leucine zipper domain) that is thought to play a role in downstream signaling of events triggered by infection and, possibly, other stresses.

The R proteins have some structural similarity to APAF-1, a protein which mediates between Bcl-2, a negative regulator of apoptosis, and caspases. A domain, designated the NB-ARC domain ("nucleotide-binding adaptor shared by APAF-1, certain R gene products and CED-4") contains a series of motifs and residues that are conserved among R proteins and APAF-1 (van der Bizen and Jones (1999) Current Biology 8:226-228). In addition to the NBS domain, APAF-1 has a CARD domain, functionally analogous to the effector domain of R proteins, and a WD-40 domain, functionally analogous to the LRR domain of R proteins.

Similar to CARD-12, CARD-4 and CARD-7 have both an NBS domain and an LRR domain as well as a CARD domain (detailed information concerning CARD-4 and CARD-7 can be found in U.S. Application Serial No. 09/245,281, filed February 5, 1999, U.S. Application Serial No. 09/207,359, filed December 8, 1998, U.S. Application Serial No. 09/099,041, filed June 17, 1998, U.S. Application Serial No. 09/019,942, filed February 6, 1998, and U.S. Application Serial No. 09/428,252, filed October 27, 1999, all of which are incorporated herein by reference). The CARD domain, which is present in a number of apoptotic signaling molecules, is an effector domain that thought to be involved in homophilic protein-protein interactions, e.g., with downstream CARD-containing signaling molecules. For example, the CARD domain of CARD-4 interacts with the CARD domain of RICK (RIP2, CARDIAK), a serine-threonine kinase that activates NF- $\kappa$ B signaling pathways.

Other proteins structurally related to CARD-12 include NBS-1, Pyrin-1, PCD-1, PCD-2, and PCD-3.

NBS-1 has an NBS domain and a LRR domain, as well as a pyrin domain. Functionally analogous to the CARD domain of CARD-4, CARD-7, and CARD-12, the pyrin domain is an effector domain thought to be involved in homophilic protein-protein interactions. Pyrin-1 also contains a pyrin domain. Detailed information concerning NBS-1 and Pyrin-1 can be found in U.S. Application Serial No. 09/506,067, filed February 17, 2000, which is incorporated herein by reference.

PCD-1, PCD-2, and PCD-3 each contain both an NBS domain and a leucine zipper domain. A leucine zipper domain, like the CARD domain and the pyrin domain, is an effector domain thought to be involved in homophilic protein-protein interactions.

PCD-2 and PCD-3 also each contains LRR domains. PCD-1, which is truncated at its carboxy terminus, is also expected to contain an LRR domain. Detailed information concerning PCD-1, PCD-2, and PCD-3 can be found in U.S. Application Serial No. 09/563,876, filed May 3, 2000, which is incorporated herein by reference.

5           In general, an NBS domain includes a kinase 1a domain (P-loop), and a kinase 2 domain (Walker B box). An LRR domain usually is composed of several leucine rich repeats.

          Without being bound by a particular theory, it is possible that CARD-12 participates in the network of interactions that lead to caspase activity. Human CARD-12  
10           may play a functional role in caspase activation similar to that of Apaf-1 (Zou et al., Cell, 90:405-413, 1997). For example, upon activation, CARD-12 might bind a nucleotide, thus allowing CARD-12 to bind and activate a CARD-containing caspase via a CARD-CARD interaction, leading to apoptotic death of the cell.

          Accordingly, CARD-12 molecules are useful as modulating agents in regulating a  
15           variety of cellular processes including cell growth and cell death. In one aspect, this invention provides isolated nucleic acid molecules encoding CARD-12 proteins or biologically active portions thereof, as well as nucleic acid fragments suitable as primers or hybridization probes for the detection of CARD-12 encoding nucleic acids.

          The invention encompasses methods of diagnosing and treating patients who are  
20           suffering from a disorder associated with an abnormal level or rate (undesirably high or undesirably low) of apoptotic cell death, abnormal activity of stress-related pathways of the endoplasmic reticulum (ER), abnormal activity of the Fas/APO-1 receptor complex, abnormal activity of the TNF receptor complex, or abnormal activity of a caspase by administering a compound that modulates the expression of CARD-12 (at the DNA,  
25           mRNA or protein level, e.g., by altering mRNA splicing) or by altering the activity of CARD-12. Examples of such compounds include small molecules, antisense nucleic acid molecules, ribozymes, and polypeptides.

          Certain disorders are associated with an increased number of surviving cells, which are produced and continue to survive or proliferate when apoptosis is inhibited or  
30           occurs at an undesirably low rate. CARD-12 and compounds that modulate the expression or activity of CARD-12 can be used to treat or diagnose such disorders. These disorders include cancer (particularly follicular lymphomas, chronic myelogenous leukemia, melanoma, colon cancer, lung carcinoma, carcinomas associated with mutations in p53, and hormone-dependent tumors such as breast cancer, prostate cancer,  
35           and ovarian cancer). Such compounds can also be used to treat viral infections (such as those caused by herpesviruses, poxviruses, and adenoviruses). Failure to remove

autoimmune cells that arise during development or that develop as a result of somatic mutation during an immune response can result in autoimmune disease. Thus, an autoimmune disorder can be caused by an undesirably low level of apoptosis. Accordingly, CARD-12 and modulators of CARD-12 activity or expression can be used to treat autoimmune disorders (e.g., systemic lupus erythematosus, immune-mediated glomerulonephritis, and arthritis).

Many diseases are associated with an undesirably high rate of apoptosis. CARD-12 and modulators of CARD-12 expression or activity can be used to treat or diagnose such disorders. A wide variety of neurological diseases are characterized by the gradual loss of specific sets of neurons. Such disorders include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), retinitis pigmentosa, spinal muscular atrophy, Huntington's disease, and various forms of cerebellar degeneration. The cell loss in these diseases does not induce an inflammatory response, and apoptosis appears to be the mechanism of cell death. In addition, a number of hematologic diseases are associated with a decreased production of blood cells. These disorders include anemia associated with chronic disease, aplastic anemia, chronic neutropenia, and the myelodysplastic syndromes. Disorders of blood cell production, such as myelodysplastic syndrome and some forms of aplastic anemia, are associated with increased apoptotic cell death within the bone marrow. These disorders could result from the activation of genes that promote apoptosis, acquired deficiencies in stromal cells or hematopoietic survival factors, or the direct effects of toxins and mediators of immune responses. Two common disorders associated with cell death are myocardial infarctions and stroke. In both disorders, cells within the central area of ischemia, which is produced in the event of acute loss of blood flow, appear to die rapidly as a result of necrosis. However, outside the central ischemic zone, cells die over a more protracted time period and morphologically appear to die by apoptosis. Additional diseases associated with an undesirably high rate of apoptosis include: ischemic and hypoxic brain injury, traumatic and excitotoxic brain damage, neuronal transplantation, acute bacterial meningitis, kidney ischemia/reperfusion injury, and liver disease. CARD-12 and modulators of CARD-12 may therefore be useful in treating and diagnosing these conditions.

Populations of cells are often depleted in the event of viral infection, with perhaps the most dramatic example being the cell depletion caused by the human immunodeficiency virus (HIV). Surprisingly, most T cells that die during HIV infections do not appear to be infected with HIV. Although a number of explanations have been proposed, recent evidence suggests that stimulation of the CD4 receptor results in the enhanced susceptibility of uninfected T cells to undergo apoptosis.

CARD-12 polypeptides, nucleic acids and modulators of CARD-12 expression or activity can be used to treat inflammatory disorders and immune system disorders. The inflammatory and immune disorders include, but are not limited to, chronic inflammatory diseases and disorders, such as Crohn's disease, reactive arthritis, including Lyme  
5 disease, insulin-dependent diabetes, organ-specific autoimmunity, including multiple sclerosis, Hashimoto's thyroiditis and Grave's disease, contact dermatitis, psoriasis, graft rejection, graft versus host disease, sarcoidosis, atopic conditions, such as asthma and allergy, including allergic rhinitis, gastrointestinal allergies, including food allergies, eosinophilia, conjunctivitis, glomerular nephritis, certain pathogen susceptibilities such as  
10 helminthic (e.g., leishmaniasis), certain viral infections, including HIV, and bacterial infections, including tuberculosis and lepromatous leprosy.

Ischemia is often accompanied by inflammation that causes cell death. Because CARD-12 is expected to play a role in stress-related response, inflammation and apoptosis, CARD-12 polypeptides, nucleic acids, and modulators of CARD-12  
15 expression or activity can be used to treat cells death accompanying inflammatory responses triggered by ischemia.

Invasive infection with Gram-negative bacteria and Gram-positive bacteria often results in septic shock. CARD-12 may recognize and bind components of Gram-negative bacteria and Gram-positive bacteria or other infectious agents (e.g., intracellular  
20 parasites), triggering an inflammatory response. Thus, CARD-12 may play a role in innate immune system responses that is similar to that of Toll-like receptor 2 (TLR2), a receptor which has some structural similarity to plant R proteins and IL-1R. TLR2 is a signaling receptor that, in association with CD14, is activated by LPS in a response that requires LPS-binding protein. The interaction of TLR2 with LPS leads to TLR2  
25 oligomerization and recruitment of IRAK (Yang et al. (1998) Nature 395:284-88; Yang et al (1999) J. Immunol. 163:639-43; and Yoshimura et al. (1999) J. Immunol. 163:105). Thus, TLR2 is thought to be a direct mediator of signaling by LPS. TLR2 is also thought to mediate cell activation induced by peptidoglycan and lipoteichoic acid, the main stimulatory components of Gram-positive bacteria (Schwandner et al. (1999) J. Biol.  
30 Chem. 274:17406-09).

In addition to the aforementioned disorders, CARD-12 polypeptides, nucleic acids, and modulators of CARD-12 expression or activity can be used to treat septic shock and other disorders associated with an innate immune response. For example, CARD-12 may bind to a component of an intracellular infectious agent or a component  
35 of an infectious agent that is brought into a cell expressing CARD-12, e.g., a component that enters a cell through a receptor or is expressed by a viral gene.

In addition to the aforementioned disorders, CARD-12 polypeptides, nucleic acids, and modulators of CARD-12 expression or activity can be used to treat disorders of cell signaling and disorders of tissues in which CARD-12 is expressed.

The invention features a nucleic acid molecule which is at least 45% (or 55%,  
5 65%, 75%, 85%, 95%, or 98%) identical to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, the nucleotide sequence of the cDNA insert of the plasmid deposited with the ATCC as Accession Number \_\_\_\_\_ (the "cDNA of ATCC \_\_\_\_\_"), or a complement thereof.

The invention features a nucleic acid molecule which includes a fragment of at  
10 least 150 (300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600, 1900, 2100, 2400, 2700, 3000, or 3100) nucleotides of the nucleotide sequence shown in SEQ ID NO:1, or SEQ ID NO:3, or the nucleotide sequence of the cDNA ATCC \_\_\_\_\_, or a complement thereof.

In an embodiment, a CARD-12 nucleic acid molecule has the nucleotide sequence  
15 shown in SEQ ID NO:1, or SEQ ID NO:3, or the nucleotide sequence of the cDNA of ATCC \_\_\_\_\_.

Also within the invention is a nucleic acid molecule which encodes a fragment of a polypeptide having the amino acid sequence of SEQ ID NO:2 or the polypeptide encoded by the cDNA of ATCC Accession Number \_\_\_\_\_. The fragment can comprise  
20 15, 25, 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 contiguous amino acids of SEQ ID NO:2.

The invention includes a nucleic acid molecule which encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule consisting  
25 of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC under stringent conditions.

In general, an allelic variant of a gene will be readily identifiable as mapping to the same chromosomal location as the gene.

The invention also includes a nucleic acid molecule encoding a naturally occurring polypeptide, wherein the nucleic acid hybridizes to a nucleic acid molecule  
30 consisting of SEQ ID NO:3 under stringent conditions (e.g., hybridization in 6X sodium chloride/sodium citrate (SSC) at about 60°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 65°C), and wherein the nucleic acid encodes a polypeptide of 1020-1028 amino acids in length, preferably 1024 amino acids, having a molecular weight of about 116.1 kD prior to post-translational modifications. Thus, the invention  
35 encompasses a nucleic acid molecule which includes the sequence of the protein coding

region of a naturally occurring mRNA (or the corresponding cDNA sequence) that is expressed in a human cell.

Also within the invention are: an isolated CARD-12 protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:2; an isolated CARD-12 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the P-loop domain of SEQ ID NO:2 (e.g., about amino acid residues 169 to 179 of SEQ ID NO:2); an isolated CARD-12 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the CARD domain of SEQ ID NO:2 (e.g., about amino acid residues 1 to 88 of SEQ ID NO:2); an isolated CARD-12 protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the nucleotide binding site core domain of SEQ ID NO:2 (e.g., about amino acid residues 161 to 323 of SEQ ID NO:2); an isolated CARD-12 protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to one or more of the leucine rich repeats of SEQ ID NO:2 (e.g., about amino acids residues 762-789, 819-846, 847-874, and 938-965 of SEQ ID NO:2); and an isolated CARD-12 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the NAIP homology region of SEQ ID NO:2 (e.g., about amino acid residues 150 to 1024 of SEQ ID NO:2).

Also within the invention are: an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence that is at least about 65%, preferably 75%, 85%, or 95% identical to SEQ ID NO:3 or the cDNA of ATCC \_\_\_\_\_; an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 85%, 95%, or 98% identical to the P-loop domain encoding portion of SEQ ID NO:3; an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical the CARD domain encoding portion of SEQ ID NO:3; an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical the NAIP homology encoding portion of SEQ ID NO:3; an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical the nucleotide binding site domain encoding portion of SEQ ID NO:3; an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical to the LRR domain encoding portion of SEQ ID NO:3 or one or more leucine rich repeat encoding portions of SEQ ID NO:3; and an isolated CARD-12 protein which is encoded by a nucleic acid molecule having a nucleotide sequence which hybridizes under

stringent hybridization conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:3 or the non-coding strand of the cDNA of ATCC \_\_\_\_\_.

The CARD-12 nucleic acids, polypeptides, and antibodies of the invention may be useful for mapping the location of the CARD-12 gene.

5 Another embodiment of the invention features CARD-12 nucleic acid molecules which specifically detect CARD-12 nucleic acid molecules, relative to nucleic acid molecules encoding other members of the CARD superfamily and/or members of the NBS/LRR superfamily. For example, in one embodiment, a CARD-12 nucleic acid molecule hybridizes under stringent conditions to a nucleic acid molecule comprising the  
10 nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC \_\_\_\_\_, or a complement thereof. In another embodiment, the CARD-12 nucleic acid molecule is at least 300 (350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600, 1900, 2100, 2400, 2700, 3000, or 3100) nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence shown in SEQ  
15 ID NO:1, SEQ ID NO:3, the cDNA of ATCC \_\_\_\_\_, or a complement thereof. In another embodiment, an isolated CARD-12 nucleic acid molecule comprises the CARD domain encoding portion of SEQ ID NO:3, or a complement thereof. In yet another embodiment, the invention provides an isolated nucleic acid molecule which is antisense to the coding strand of a CARD-12 nucleic acid.

20 Another aspect of the invention provides a vector, e.g., a recombinant expression vector, comprising a CARD-12 nucleic acid molecule of the invention. In another embodiment the invention provides a host cell containing such a vector. The invention also provides a method for producing CARD-12 protein by culturing, in a suitable medium, a host cell of the invention containing a recombinant expression vector such that  
25 a CARD-12 protein is produced.

Another aspect of this invention features isolated or recombinant CARD-12 proteins and polypeptides. Preferred CARD-12 proteins and polypeptides possess at least one biological activity possessed by naturally occurring human CARD-12, e.g., (1) the ability to form protein:protein interactions with proteins in the apoptotic signaling  
30 pathway; (2) the ability to form CARD-CARD interactions with proteins in the apoptotic signaling pathway; (3) the ability to bind a CARD-12 ligand; and (4) the ability to bind to an intracellular target. Other activities include: (1) modulation of cellular proliferation; (2) modulation of cellular differentiation; (3) modulation of cellular death; (4) modulation of ER-specific apoptosis pathways; (5) modulation of the NF-kB pathway;  
35 (6) modulation of stress-responsive signaling pathways; and (7) modulation of an innate immune response.

The CARD-12 proteins of the present invention, or biologically active portions thereof, can be operatively linked to a non-CARD-12 polypeptide (e.g., heterologous amino acid sequences) to form CARD-12 fusion proteins, respectively. The invention further features antibodies that specifically bind CARD-12 proteins, such as monoclonal  
5 or polyclonal antibodies. In addition, the CARD-12 proteins or biologically active portions thereof can be incorporated into pharmaceutical compositions, which optionally include pharmaceutically acceptable carriers.

In another aspect, the present invention provides a method for detecting the presence of CARD-12 activity or expression in a biological sample by contacting the  
10 biological sample with an agent capable of detecting an indicator of CARD-12 activity such that the presence of CARD-12 activity is detected in the biological sample.

In another aspect, the invention provides a method for modulating CARD-12 activity comprising contacting a cell with an agent that modulates (inhibits or stimulates) CARD-12 activity or expression such that CARD-12 activity or expression in the cell is  
15 modulated. In one embodiment, the agent is an antibody that specifically binds to CARD-12 protein. In another embodiment, the agent modulates expression of CARD-12 by modulating transcription of a CARD-12 gene, splicing of a CARD-12 mRNA, or translation of a CARD-12 mRNA. In yet another embodiment, the agent is a nucleic acid molecule having a nucleotide sequence that is antisense to the coding strand of the  
20 CARD-12 mRNA or the CARD-12 gene.

In one embodiment, the methods of the present invention are used to treat a subject having a disorder characterized by aberrant CARD-12 protein or nucleic acid expression or activity or related to CARD-12 expression or activity by administering an agent which is a CARD-12 modulator to the subject. In one embodiment, the CARD-12  
25 modulator is a CARD-12 protein. In another embodiment the CARD-12 modulator is a CARD-12 nucleic acid molecule. In other embodiments, the CARD-12 modulator is a peptide, peptidomimetic, or other small molecule.

The present invention also provides a diagnostic assay for identifying the presence or absence of a genetic lesion or mutation characterized by at least one of: (i)  
30 aberrant modification or mutation of a gene encoding a CARD-12 protein; (ii) mis-regulation of a gene encoding a CARD-12 protein; (iii) aberrant RNA splicing; and (iv) aberrant post-translational modification of a CARD-12 protein, wherein a wild-type form of the gene encodes a protein with a CARD-12 activity.

In another aspect, the invention provides a method for identifying a compound  
35 that binds to or modulates the activity of a CARD-12 protein. In general, such methods entail measuring a biological activity of a CARD-12 protein in the presence and absence



of a test compound and identifying those compounds which alter the activity of the CARD-12 protein.

5 The invention also features methods for identifying a compound which modulates the expression of CARD-12 by measuring the expression of CARD-12 in the presence and absence of a compound.

The invention also features methods for treating disorders associated with inappropriate apoptosis (e.g., Alzheimer's diseases or other neurological disorders associated with neuronal apoptosis) by modulating the expression or activity of CARD-12.

10 The invention also features methods for identifying a compound that alters (increases or decreases) the binding of CARD-12 (or a CARD domain containing portion thereof) to a CARD domain containing protein (e.g., CARD-5) or a CARD domain containing portion thereof. The method includes measuring the binding of the protein (or polypeptides) to each other in the presence and absence of a test compound and  
15 identifying the test compound as a compound that alters binding if the binding in the presence of test compound differs from the binding in the absence of the test compound.

The invention also features a method for identifying a compound that binds to the NBS domain of CARD-12 by measuring the binding of a test compound to a polypeptide comprising the NBS domain of CARD-12. The binding can be measured in the presence  
20 of a nucleotide (e.g., an NTP such as ATP) for a competitive binding assay. Alternatively, the binding can be measured in the absence of a nucleotide that binds to the NBS site.

The invention also features a method for identifying compounds that alter (increase or decrease) CARD-12 mediated apoptosis. The methods include measuring  
25 apoptosis in the presence and absence of a test compound in cells expressing CARD-12 and in cells not expressing CARD-12 (or expressing less CARD-12). The CARD-12 expressed by the cell can be encoded by a vector introduced into the cell. Thus, the cells can over-express CARD-12. A compound that alters apoptosis in the cells expressing CARD-12, but not in the cells not expressing CARD-12 (or expressing less CARD-12),  
30 the compound is a candidate CARD-12 specific modulator of apoptosis.

Compounds that increase the binding of CARD-12 to CARD-5 can be used to supplement chemotherapeutic agents.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

35

### Brief Description of the Drawings

Figures 1A-1E depict the cDNA sequence (SEQ ID NO:1) and predicted amino acid sequence (SEQ ID NO:2) of human CARD-12. The open reading frame of CARD-12 (SEQ ID NO:1) extends from nucleotide 36 to nucleotide 3107 of SEQ ID NO:1 (SEQ ID NO:3).

Figures 2A-2E depict a predicted nucleotide sequence (SEQ ID NO:4) and predicted amino acid sequence (SEQ ID NO:5) of human CARD-12, based upon the CARD-12 sequence derived from a genomic clone.

Figure 3 depicts a hydropathy plot of CARD-12. Relatively hydrophobic residues are above the dashed horizontal line, and relatively hydrophilic residues are below the dashed horizontal line. The cysteine residues (cys) and N-glycosylation (Ngly) site are indicated by short vertical lines just below the hydropathy trace.

Figure 4 depicts a plot showing the predicted structural features of CARD-12. This figure shows the predicted alpha regions (Garnier-Robson and Chou-Fasman), the predicted beta regions (Garnier-Robson and Chou-Fasman), the predicted turn regions (Garnier-Robson and Chou-Fasman) and the predicted coil regions (Garnier-Robson). Also included in the figure is a hydrophilicity plot (Kyte-Doolittle), the predicted alpha and beta-amphipathic regions (Eisenberg), the predicted flexible regions (Karplus-Schulz), the predicted antigenic index (Jameson-Wolf) and the predicted surface probability plot (Emini).

Figure 5A depicts an alignment of amino acids 2-88 of human CARD-12 (amino acid residues 2-88 of SEQ ID NO:2) with a CARD domain (SEQ ID NO:7) derived from a hidden Markov model.

Figure 5B depicts an alignment of amino acids 764-791 of human CARD-12 (amino acid residues 764-791 of SEQ ID NO:2) with a consensus leucine rich repeat (SEQ ID NO:8) derived from a hidden Markov model.

Figure 5C depicts an alignment of amino acids 821-848 of human CARD-12 (amino acid residues 821-848 of SEQ ID NO:2) with a consensus leucine rich repeat (SEQ ID NO:8) derived from a hidden Markov model.

Figure 5D depicts an alignment of amino acids 849-872 of human CARD-12 (amino acid residues 849-872 of SEQ ID NO:2) with a consensus leucine rich repeat (SEQ ID NO:8) derived from a hidden Markov model.

Figure 5E depicts an alignment of amino acids 938-965 of human CARD-12 (amino acid residues 938-965 of SEQ ID NO:2) with a consensus leucine rich repeat (SEQ ID NO:8) derived from a hidden Markov model.

Figures 6A-6C depict an alignment of amino acids 150-1024 of human CARD-12 (amino acid residues 150-1024 of SEQ ID NO:2) with amino acids 451-1232 of neuronal AIP (SEQ ID NO:9).

Figure 7 depicts an alignment of the CARD domain of human CARD-12 (amino acids 1-88 of SEQ ID NO:2) with the CARD domains of CARD-4, CARD-7, and Apaf-1.

Figure 8 depicts schematic drawings of the domain structures of human CARD-12, human CARD-4, human CARD-7, human Nod2, and Apaf-1.

Figure 9 depicts the results of a mammalian two-hybrid assay used to identify CARD domains that interact with the CARD domain of CARD-12.

Figure 10 depicts the results of co-immunoprecipitation analysis. Panel A: After 24 hrs, extracts were prepared and immunoprecipitated (IP) with a monoclonal antibody to the T7 epitope. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with an anti-myc polyclonal antibody. Panel B: The cellular extracts were also immunoblotted (WB) with anti-T7 antibody. Panel C: Extracts were also immunoprecipitated with a monoclonal antibody to myc, followed by WB with an anti-myc polyclonal antibody.

#### Detailed Description of the Invention

The present invention is based, in part, on the identification of a cDNA sequence encoding a human CARD-12 protein. A nucleotide sequence encoding a human CARD-12 protein is shown in Figures 1A-1E (SEQ ID NO:1; SEQ ID NO:3 includes the open reading frame only). A predicted amino acid sequence of CARD-12 protein is also shown in Figures 1A-1E (SEQ ID NO:2).

#### Example 1: Identification and Characterization of CARD-12

A BAC clone (GenBank™ Accession Number AL121653) was searched to identify potential exons. The predicted protein sequences were then searched using a profile of known CARD domains. This search led to the identification of a sequence predicted to encode a CARD domain-containing protein later identified as CARD-12. Figures 2A-2E depict a nucleotide sequence (SEQ ID NO:4) assembled from the BAC clone which includes a predicted open reading frame (SEQ ID NO:6; nucleotides 1-3612 of SEQ ID NO:4) encoding a 1204 amino acid protein (SEQ ID NO:5).

The CARD-12 sequence assembled from the BAC clone was used to further characterize the CARD-12 cDNA sequence. A search of a proprietary electronic cDNA database (created from a human lymphocyte library) using sequences derived from the 3' end of SEQ ID NO:4 led to the identification of a cDNA containing the 3' end of the

CARD-12 cDNA. The sequence of the 5' end of the CARD-12 cDNA was determined by a search of the Incyte (Palo Alto, CA) Life Gold Templates cDNA electronic database using sequences from the 5' end of SEQ ID NO:4. A cDNA sequence was identified (Incyte clone number 328193) that contained 366 nucleotides of the 5' portion of the CARD-12 cDNA. Primers were designed corresponding to the 5' untranslated region and the 3' carboxy terminus coding region of CARD-12. PCR amplification using these primers was performed on a placenta cDNA library. The sequencing of PCR products led to the identification of the cDNA sequence of CARD-12 (SEQ ID NO:1).

The CARD-12 cDNA sequence (SEQ ID NO:1) has some differences compared to that predicted by the assembly of the predicted CARD-12 exons of the BAC clone (SEQ ID NO:4). These differences are generally located at the 5' and 3' end of the cDNA. Where the CARD-12 sequences depicted in SEQ ID NO:1 and SEQ ID NO:4 differ, SEQ ID NO:1 corresponds to the CARD-12 cDNA sequence.

Figures 1A-1E depict the sequence of a 3133 nucleotide cDNA (SEQ ID NO:1) which includes a predicted open reading frame (SEQ ID NO:3; nucleotides 36-3107 of SEQ ID NO:1) encoding a 1024 amino acid human CARD-12 protein (SEQ ID NO:2). Human CARD-12 is predicted to be an intracellular protein.

The predicted amino acid sequence of human CARD-12 was compared to amino acid sequences of known proteins and various motifs were identified. The 1024 amino acid human CARD-12 protein includes two N-glycosylation sites (e.g., about amino acid residues 539-542 and 764-767 of SEQ ID NO:2); a glycosaminoglycan attachment site (e.g., about amino acid residues 171-174 of SEQ ID NO:2); four cAMP- and cGMP-dependent protein kinase phosphorylation site (e.g., about amino acid residues 60-63, 228-231, 453-456, and 985-988 of SEQ ID NO:2); 11 protein kinase C phosphorylation sites (e.g., about amino acid residues 72-74, 171-173, 188-190, 226-228, 403-405, 536-538, 566-568, 665-667, 689-691, 710-712, and 973-975 of SEQ ID NO:2); 20 casein kinase II phosphorylation sites (e.g., about amino acid residues 72-75, 94-97, 133-136, 215-218, 279-282, 365-368, 415-418, 445-448, 460-463, 479-482, 497-500, 541-544, 553-556, 607-610, 665-668, 725-728, 742-745, 851-854, 920-923, and 973-976 of SEQ ID NO:2); a tyrosine kinase phosphorylation site (e.g., about amino acid residues 71-78 of SEQ ID NO:2); 15 N-myristoylation sites (e.g., about amino acid residues 62-67, 156-161, 187-192, 211-216, 291-296, 380-385, 516-521, 618-623, 699-704, 754-759, 760-765, 894-899, 928-933, 946-951, and 959-964 of SEQ ID NO:2); an amidation site (e.g., about amino acid residues 451-454 of SEQ ID NO:2); and an ATP/GTP-binding site motif A (P-loop) (e.g., about amino acid residues 169-179 of SEQ ID NO:2).

Figure 3 depicts a hydropathy plot of CARD-12. Relatively hydrophobic residues are above the dashed horizontal line, and relatively hydrophilic residues are below the dashed horizontal line. The cysteine residues (cys) and N-glycosylation (Ngly) site are indicated by short vertical lines just below the hydropathy trace.

5 A plot showing the predicted structural features of CARD-12 is presented in Figure 4. This figure shows the predicted alpha regions (Garnier-Robson and Chou-Fasman), the predicted beta regions (Garnier-Robson and Chou-Fasman), the predicted turn regions (Garnier-Robson and Chou-Fasman) and the predicted coil regions (Garnier-Robson). Also included in the figure is a hydrophilicity plot (Kyte-Doolittle),  
10 the predicted alpha and beta-amphipathic regions (Eisenberg), the predicted flexible regions (Karplus-Schulz), the predicted antigenic index (Jameson-Wolf) and the predicted surface probability plot (Emini).

An analysis of the predicted CARD-12 amino acid sequence showed it to contain a CARD domain (e.g., about amino acid residues 1-88 of SEQ ID NO:2), a nucleotide  
15 binding site (NBS) domain (e.g., about amino acid residues 161-323 of SEQ ID NO:2), and three leucine rich repeats (LRR; e.g., about amino acid residues 762-789, 819-846, 847-874, and 938-965 of SEQ ID NO:2) which form a LRR domain (e.g., about amino acid residues 762-965 of SEQ ID NO:2). Within the predicted NBS domain there is a kinase 1a domain (P-loop) (e.g., about amino acid residues 169-179 of SEQ ID NO:2)  
20 and a kinase 2 domains (e.g., about amino acid residues 245-248 of SEQ ID NO:2).

Figure 5A depicts an alignment of amino acids 2-88 of human CARD-12 (amino acid residues 2-88 of SEQ ID NO:2) with a consensus CARD domain (SEQ ID NO:7) derived from a HMM.

Figures 5B-5E each depict an alignment of one of the four leucine rich repeats  
25 within the LRR domain of CARD-12 (amino acid residues 764-791 of SEQ ID NO:2 (Figure 5B), amino acid residues 821-848 of SEQ ID NO:2 (Figure 5C), amino acid residues 849-872 of SEQ ID NO:2 (Figure 5D), and amino acid residues 938-965 of SEQ ID NO:2 (Figure 5E)) with a consensus LRR (SEQ ID NO:8) derived from a HMM.

The domain alignments depicted in Figures 5A-5E were identified by homology  
30 searching using consensus domains derived from hidden Markov models (HMMs). HMMs can be used to perform multiple sequence alignment and very sensitive database searching, using statistical descriptions of a domain's consensus sequence. For more information on HMM searches, see, e.g., <http://hmmer.wustl.edu/>. In the alignments of Figures 5A-5E a single letter amino acid designation at a position on the line between the  
35 CARD-12 sequence and the HMM-generated consensus domain sequence indicates an exact match between the two. A "+" in this middle line indicates a conservative

substitution at the particular residue of CARD-12. Amino acid residues located in the domains identified by the HMM search may be important for the appropriate functioning of the CARD-12 protein. For this reason, amino acid substitutions with respect to the sequence of SEQ ID NO:2 that are outside of the domains homologous to HMM consensus domains may be less detrimental to the activity of the CARD-12 protein.

The C-terminal portion of CARD-12 (amino acids 150 to 1024 of SEQ ID NO:2) bears some similarity to the N-terminus of neuronal AIP (GenBank™ Accession Number Q13075; Roy et al. (1995) Cell 80:167-178). Figures 6A-6C depict an alignment of amino acids 451-1232 of neuronal AIP (SEQ ID NO:9) and amino acids 150-1024 of human CARD-12.

#### Example 2: Additional Characterization of CARD-12 Domains

CARD-12 includes seven NACHT (NAIP, CIIA, HET-E and TP1) NTPase domains. The seven NACHT NTPase domains are at amino acids 169-186 of SEQ ID NO:2 (P-Loop/Walker A Box /Motif I); amino acids 196-220 of SEQ ID NO:2 (Walker B Box/Mg<sup>++</sup> binding domain Motif II); amino acids 229-253 of SEQ ID NO:2 (Motif III); amino acids 261-282 of SEQ ID NO:2 (Motif IV); amino acids 330-351 of SEQ ID NO:2 (Motif V); amino acids 414-430 of SEQ ID NO:2 (Motif VI) and amino acids 438-457 of SEQ ID NO:2 (Motif VII). Other members of the NACHT NTPase family include: CARD-4, CARD-7, and NAIP (Koonin et al. (2001) Trends Biochem. Sci. 25:223).

Additional analysis of the leucine-rich repeat domain of CARD-12 revealed that the domain extends from amino acid 656-1021 of SEQ ID NO:2 and contains 13 leucine-rich repeats (amino acids 656-686, 687-708, 711-737, 738-761, 762-788, 789-817, 819-845, 846-874, 875-901, 903-930, 936-962, 965-993, and 994-1021 of SEQ ID NO:2).

Additional analysis of CARD-12 revealed that the nucleotide binding site domain extends from amino acid 169 to amino acid 456 of SEQ ID NO:2.

As discussed above, the CARD domain of CARD-12 extends from amino acid 1-88 of SEQ ID NO:2. Figure 7 depicts an alignment of the CARD domain of human CARD-12 (amino acids 1-88 of SEQ ID NO:2) with the CARD domains of CARD-4, CARD-7, and Apaf-1.

As discussed above, CARD-12 includes domains present in members of the CED/Apaf-1 family. Figure 8 depicts schematic drawings of the domain structures of human CARD-12 as well as human CARD-4, CARD-7, Nod2, and Apaf-1, all of which are members of the CED/Apaf-1 family.

Molecules that binding to and alter the activity of the NBS domain of CARD-12 may be useful for modulating the activity of CARD-12. For example, molecules can be

tested for their ability to modulate, e.g., antagonize, the hydrolysis of an NTP, e.g., ATP, by the nucleotide-binding site of CARD-12. Methods of detecting the hydrolysis of ATP by a nucleotide-binding site are described in, for example, Gadsby et al. (1999) *Physiol. Rev.* 79:S77-S107. Additional assays that might be used are described in Li et al., (1996) *J. Biol. Chem.* 271: 28463-28468.

### Example 3: Expression Analysis

Northern blot analysis of CARD-12 expression using a human adult tissue blot (Clontech, La Jolla, CA) revealed that a 3.3-kilobase CARD-12 transcript is present in human lymphoid tissues, including spleen, peripheral blood lymphocytes, bone marrow and fetal liver.

An affinity purified polyclonal CARD-12 antibody was used to investigate expression in primary normal human epithelial cells (Epipanel; Clonetics, Inc.). Affinity-purified CARD-12 antibody was raised in rabbits injected with the 15-mer peptide LWRQESLQSVKNTTE corresponding to residues 527-542 of CARD-12 (Research Genetics). The analysis revealed that a about 120 kD CARD-12 protein is expressed in human renal cortical primary epithelial cells, human mammary primary epithelial cells, human renal proximal tubule primary epithelial cells, human bronchial primary epithelial cells, and human prostate primary epithelial cells. Expression was also detected in human primary epithelial cells, and muscle primary cells.

### Example 4: CARD-12 Mediates Apoptosis

To determine a possible role for CARD-12 in apoptosis signaling a recombinant adenovirus expressing full length CARD-12 was constructed. Briefly, CARD-12 was expressed using a dual-adenovirus based, tetracycline-regulatable expression system. A similar system has previously been shown to work extremely efficiently both *in vitro* and *in vivo*. CARD-12 was cloned into the adenovirus transfer vector pLE11f, placing the CARD-12 gene under the transcriptional control of the tetracycline-regulatable promoter. An internal downstream ribosome entry site allowed a modified green fluorescent protein (KGFP) to be expressed off the same transcript. E1/E3-deleted adenovirus was then generated by homologous recombination in 911 cells (Fallaux et al. (1996) *Hum. Gene Ther.* 7:215), plaque-purified and protein expression verified by Western blot. VERO cells were transfected at an MOI of about 20. As a control, cells were similarly transfected with an adenovirus vector expressing KGFP only. VERO cells were plated in 96 well dishes and transfected the following day with adenovirus (MOI of about 20).

Cells were fixed (4% paraformaldehyde in 0.15M PBS) 36 hours after transfection. The nuclei were then stained with Hoescht 3342 and the percentage of apoptotic versus healthy nuclei in transfected cells was scored. Within 36 hours of transfection with CARD-12 expressing adenovirus, the transfected cells were undergoing apoptosis, as indicated by rounding up and membrane blebbing. Additionally,  $45.2 \pm 4.0\%$  (mean  $\pm$  SE) of CARD-12-transfected cells had condensed, pyknotic nuclei, whereas only  $3.8 \pm 1.1\%$  of KGFP-transfected cells showed signs of apoptosis. Adenovirus-mediated transfection of either CARD-12 or KGFP in VERO cells resulted in at least 90% of cells transfected. Thus, CARD-12 is the novel member of the Apaf-1/CED4 protein family (along with CARD-4, CARD-7, Nod2 and Apaf-1) shown to activate downstream cell death signals. As a member of the Apaf-1/CED4 family, transduction of a proapoptotic signal through CARD-12 is likely to be mediated by CARD/CARD interaction with one or more CARD-containing signaling molecules.

#### Example 5: CARD-12 Interacts with CARD-5 (ACS)

A mammalian two-hybrid analysis was used to identify potential binding partners of CARD-12. In this analysis the binding of the N-terminal CARD of CARD-12 to the CARD domains of 23 known proteins was assessed. The analysis involved the use of a plasmid, pCMV-CARD-12-CARD/AD, constructed by inserting the CARD domain of CARD-12 (residues 1-83) into pCMV-AD (Stratagene; La Jolla, CA). In the assay, 293T cells in 6-well plates (35-mm wells) were transfected with the following plasmids: 750 ng of pCMV-CARD-12/AD, 750ng of pCMV-BD fused to individual CARD domains, 250ng of pFR-Luc firefly reporter (Stratagene), and 250ng of pRL-TK renilla reporter (Promega). Cells were harvested 24 hrs after transfection, and firefly luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). In addition, renilla luciferase activity was determined and used to normalize transfection efficiencies. The results of the two-hybrid analysis are shown in Figure 9. The CARD of CARD-12 interacted with the CARD of CARD-5, resulting in an 10-fold increase in relative luciferase activity. The CARD domain of CARD-12 also had a strong self-association, resulting in a 30-fold increase in relative luciferase activity (Figure 9). Co-expression of CARD-12 CARD with other CARD domains failed to activate luciferase expression indicating that the CARD of CARD-12 interacts selectively with the CARD of CARD-5, a protein that mediates apoptosis induced by chemotherapeutic agents (Masumoto et al. (1999) J. Biol. Chem. 274:33835).



#### Example 6: The Pyrin Domain of CARD-5 Mediates Apoptosis

CARD-5, which consists of an N-terminal PYRIN domain and a C-terminal CARD domain is a proapoptotic protein and is subject to methylation-induced silencing in a number of breast cancers. This, latter observation suggests that CARD-5 may play a  
5 fundamental role in cell death. Given that CARD-12 and CARD-5 interact via their respective CARDS, the PYRIN domain of CARD-5 may function as its proapoptotic effector domain. To examine this possibility, adenovirus vectors expressing CARD-5 truncation mutants were created. Briefly, CARD-5 truncation mutants containing the PYRIN domain or the CARD domain, were cloned into the adenovirus transfer vector  
10 pLE11f, placing the gene of interest under the transcriptional control of the tetracycline-regulatable promoter. An internal ribosome entry site downstream to the gene of interest allows a modified KGFP to be expressed off the same transcript. E1/E3-deleted adenovirus was then generated by homologous recombination in 911 cells, plaque-purified and protein expression verified by Western blot. VERO cells were transfected  
15 (MOI=20) with recombinant adenovirus expressing full length CARD-5 (AdTRE-CARD51-195), or either of the PYRIN domain (AdTRE-CARD-5-PYR1-150) or CARD (AdTRE-CARD-5-CARD74-195) of CARD-5 alone. Thirty-six hours after transfection cells were fixed and stained with the nuclear dye Hoescht 33342 and the percentage of apoptotic versus healthy nuclei in transfected cells was then scored. Western blot for the  
20 FLAG epitope-tag indicate relative levels of expression from each vector. Thirty-six hours after transfection of VERO cells with an adenovirus expressing full length CARD-5 60.4  $\pm$  1.6% of cells were undergoing apoptosis. Interestingly, transfection with the PYRIN domain of CARD-5 alone resulted 66.1  $\pm$  5.4% cell death. Expression of the CARD of CARD-5 alone resulted in virtually no cell death (2.6  $\pm$  0.6%). These results  
25 suggest that a PYRIN domain can play a functional role in apoptosis signaling, and substantiates the emerging hypothesis that PYRIN-containing proteins represent another important family of proteins involved in transducing the complex signals of apoptosis.

#### Example 7: CARD-12 Interacts with Caspase-1

30 An additional mechanism by which CARD-12 may cause cell death is by the activation of upstream caspases via a CARD/CARD interaction. The proform of caspase-1 and caspase-9 both contain an N-terminal CARD. Therefore, caspase-1 and caspase-9 were investigated as possible CARD-12 signaling partners following transient overexpression in cells. Briefly, 293T cells transfected with plasmids were lysed in  
35 50mM Tris, pH 8.0, 120mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40 buffer and incubated with either a T7 (Sigma) or myc monoclonal antibody (SantaCruz

Biotechnology, Inc.). The immune complexes were precipitated with protein G-Sepharose (Amersham Pharmacia Bio), washed extensively, and then subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with polyclonal antibody to myc (SantaCruz). The results of the co-immunoprecipitation analysis are shown in Figure 10.

5 Immunoprecipitation of T7-tagged caspase-1, but not T7-tagged procaspase-9, co-precipitated myc-tagged CARD-12 (Figure 10, Panel A, lanes 1 and 3, respectively). These results indicate a possible role for CARD-12 in caspase-1 signaling. The structural similarity of CARD-12 with other members of the Apaf-1/CED4 family members suggests a possible mechanism for its proapoptotic activity. In the case of both CARD-4 and Apaf-1, upstream signal-induced self-oligomerization at the central NBS domain leads to the induced proximity of effector molecules bound in heterotypic CARD/CARD interactions. Apaf-1 interacts with procaspase-9 via a CARD/CARD interaction. Oligomerization of Apaf-1 in response to mitochondrial cytochrome-c release induces proximity of bound procaspase-9 molecules, which then autoactivate, leading to downstream activation of caspase-3. This "Induced Proximity Model" of protein activation has been proposed as a general mechanism of caspase activation and of signal transduction for signaling partners such as CARD-4/RICK, Apaf-1/Caspase-9 and FADD/Caspase-8. Based on the structural and functional data presented here, it is reasonable to speculate that the proapoptotic activity of CARD-12 may occur through oligomerization of CARD-12 molecules at the central NBS domain in response to an upstream stress signal. CARD-12-induced cell death may then proceed in a CARD-5-dependent or possibly caspase-1 dependent path. Similarly, CARD-12 may be involved in proinflammatory signaling by influencing caspase-1 activation of interleukin-1beta.

**TABLE 1: Summary of Human CARD-12 Sequence Information**

Source of CARD-12 Sequence	Predicted cDNA	Predicted Protein	Predicted ORF	Figure
cDNA sequence	SEQ ID NO:1	SEQ ID NO:2	SEQ ID NO:3	Figs. 1A-1E
genomic sequence	SEQ ID NO:4	SEQ ID NO:5	SEQ ID NO:6	Figs. 2A-2E

**TABLE 2: Summary of Domains of CARD-12**

<b>Domain</b>	<b>Location in CARD-12</b>
<b>CARD</b>	about amino acids 1-88 of SEQ ID NO:2
<b>NBS</b>	about amino acids 169-456 of SEQ ID NO:2
<b>NACHT</b>	about amino acids 169-186 (P-Loop/Walker A Box/Motif I); 196-220 (Walker B Box/Motif II); 229-253 (Motif III); 261-282 (Motif IV); 330-351 (Motif V); 414-430 (Motif VI) and 438-457 (Motif VII) of SEQ ID NO:2
<b>Neuronal AIP Homology</b>	about amino acids 150-1024 of SEQ ID NO:2
<b>Leucine rich repeats</b>	about amino acids 656-686, 687-708, 711-737, 738-761, 762-788, 789-817, 819-845, 846-874, 875-901, 903-930, 936-962, 965-993, and 994-1021 of SEQ ID NO:2.
<b>LRR Domain</b>	about amino acids 656-1021 of SEQ ID NO:2

5 A plasmid containing a \_\_\_ encoding human CARD-12 (p\_\_\_) was deposited  
 with the American Type Culture Collection (ATCC), Manassas, VA on \_\_\_, 1999, and  
 assigned Accession Number \_\_\_\_\_. This deposit will be maintained under the terms of  
 the Budapest Treaty on the International Recognition of the Deposit of Microorganisms  
 for the Purposes of Patent Procedure. This deposit was made merely as a convenience for  
 those of skill in the art and is not an admission that a deposit is required under 35 U.S.C.  
 10 §112.

A region, the CARD domain, of human CARD-12 protein (SEQ ID NO:2) bears  
 some similarity to the CARD domains of CARD-3, CARD-4, CARD-5, CARD-6,  
 CARD-7, CARD-8, CARD-9, CARD-10, CARD-11, CARD-13, CARD-14, and CARD-  
 15 CARD-15. Detailed information concerning CARD-3, CARD-4, CARD-5, CARD-6, CARD-7,  
 CARD-8, CARD-9, CARD-10, CARD-11, CARD-13, CARD-14, and CARD-15, can be  
 found in U.S. Application Serial No. 09/245,281, filed February 5, 1999, U.S.  
 Application Serial No. 09/207,359, filed December 8, 1998, U.S. Application Serial No.  
 09/099,041, filed June 17, 1998, U.S. Application Serial No. 09/019,942, filed February  
 6, 1998, U.S. Application Serial No. 09/428,252, filed October 27, 1999, U.S.  
 20 Application Serial No. 60/180,021, filed February 3, 2000, U.S. Application Serial No.  
 09/573,641, filed May 17, 2000, U.S. Application Serial No. 60/181,159 filed February 9,

2000, U.S. Application Serial No. 60/168,780 filed December 3, 1999, U.S. Application Serial No. 09/507,533 filed February 18, 2000, and U.S. Application Serial No. 09/513,904 filed February 25, 2000. The entire content of each of these applications is incorporated herein by reference.

5 Human CARD-12 is a member of a family of molecules (the CARD-12 family) having certain conserved structural and functional features. The term "family" when referring to the protein and nucleic acid molecules of the invention is intended to mean two or more proteins or nucleic acid molecules having a common structural domain and having sufficient amino acid or nucleotide sequence identity as defined herein. Such  
10 family members can be naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin and a homologue of that protein of murine origin, as well as a second, distinct protein of human origin and a murine homologue of that protein. Members of a family may also have common functional characteristics.

15 Preferred CARD-12 polypeptides of the present invention include an amino acid sequence sufficiently identical to one or more of the following domains: a CARD domain, an NBS domain, and a LRR domain.

As used herein, the term "sufficiently identical" refers to a first amino acid or nucleotide sequence which contains a sufficient or minimum number of identical or  
20 equivalent (e.g., an amino acid residue which has a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have a common structural domain and/or common functional activity. For example, amino acid or nucleotide sequences which contain a common structural domain having about 65% identity, preferably 75% identity,  
25 more preferably 85%, 95%, or 98% identity are defined herein as sufficiently identical.

As used interchangeably herein a "CARD-12 activity", "biological activity of CARD-12" or "functional activity of CARD-12", refers to an activity exerted by a CARD-12 protein, polypeptide or nucleic acid molecule on a CARD-12 responsive cell as determined in vivo, or in vitro, according to standard techniques. CARD-12 may act  
30 as a pro-apoptotic protein or an anti-apoptotic protein (i.e., it might act to decrease or increase apoptosis). A CARD-12 activity can be a direct activity, such as an association with or an enzymatic activity on a second protein or an indirect activity, such as a cellular signaling activity mediated by interaction of the CARD-12 protein with a second protein.

In one embodiment, a CARD-12 activity can include at least one or more of the  
35 following activities: (i) the ability to interact with proteins in an apoptotic signaling pathway (ii) the ability to interact with a CARD-12 ligand; or (iii) the ability to interact

with an intracellular target protein; (iv) the ability to interact, directly or indirectly with one or more with proteins having a CARD domain, e.g., a caspase or an AIP (e.g., AIP-1 or AIP-2); (v) the ability to modulate the activity of a caspase, e.g., caspase-9; (vi) the ability to modulate the activity of NF- $\kappa$ B; (vii) the ability to modulate Apaf-1; (viii) the ability to interact directly or indirectly with a Bcl-2 family member; (ix) the ability to modulate the activity of a stress activated kinase (e.g., JNK/p38); and (x) the ability to modulate phosphorylation of CHOP (GADD 153). CARD-12 nucleic acid and polypeptides as well as modulators of activity of expression of CARD-12 might be used to modulate an Apaf-1 signaling pathway. CARD-12 may modulate the activity of a neurotrophin receptor and thus modulate apoptosis of neuronal cells. Accordingly, CARD-12 nucleic acids and polypeptides as well as modulators of CARD-12 activity or expression can be used to modulate apoptosis of neurons (e.g., for treatment of neurological disorders, particularly neurodegenerative disorders).

Accordingly, another embodiment of the invention features isolated CARD-12 proteins and polypeptides having a CARD-12 activity.

Various aspects of the invention are described in further detail in the following subsections.

### **I. Isolated Nucleic Acid Molecules**

One aspect of the invention pertains to isolated nucleic acid molecules that encode CARD-12 proteins or biologically active portions thereof, as well as nucleic acid molecules sufficient for use as hybridization probes to identify CARD-12-encoding nucleic acids (e.g., CARD-12 mRNA) and fragments for use as PCR primers for the amplification or mutation of CARD-12 nucleic acid molecules. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated CARD-12 nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of

the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

5           A nucleic acid molecule of the present invention, e.g., a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC \_\_\_\_\_, or a complement of any of these nucleotide sequences, can be isolated using standard molecular biology techniques and the sequence information provided herein. Using all or portion of the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:3, the  
10       cDNA of ATCC \_\_\_\_\_, as a hybridization probe, CARD-12 nucleic acid molecules can be isolated using standard hybridization and cloning techniques (e.g., as described in Sambrook et al., eds., *Molecular Cloning: A Laboratory Manual*. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

15           A nucleic acid of the invention can be amplified using cDNA, mRNA or genomic DNA as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to CARD-12 nucleotide sequences can be prepared by standard synthetic  
20       techniques, e.g., using an automated DNA synthesizer.

          In another embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC \_\_\_\_\_, or a portion thereof. A nucleic acid molecule which is complementary to a given nucleotide sequence  
25       is one which is sufficiently complementary to the given nucleotide sequence that it can hybridize to the given nucleotide sequence thereby forming a stable duplex.

          Moreover, the nucleic acid molecule of the invention can comprise only a portion of a nucleic acid sequence encoding CARD-12, for example, a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of CARD-  
30       12. The nucleotide sequence determined from the cloning of the human CARD-12 gene allows for the generation of probes and primers designed for use in identifying and/or cloning CARD-12 homologues in other cell types, e.g., from other tissues, as well as CARD-12 homologues and orthologs from other mammals. The probe/primer typically comprises substantially purified oligonucleotide. The oligonucleotide typically  
35       comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably about 50, 75, 100, 125, 150, 175,

200, 250, 300, 350 or 400 consecutive nucleotides of the sense or anti-sense sequence of SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC \_\_\_\_\_, or of a naturally occurring mutant of one of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC \_\_\_\_\_.

Probes based on the CARD-12 nucleotide sequence can be used to detect  
5 transcripts or genomic sequences encoding the same or similar proteins. The probe comprises a label group attached thereto, e.g., a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying allelic variants and orthologs of the CARD-12 proteins of the present invention, identifying cells or tissue which mis-express a CARD-12 protein, such as by  
10 measuring a level of a CARD-12-encoding nucleic acid in a sample of cells from a subject, e.g., detecting CARD-12 mRNA levels or determining whether a genomic CARD-12 gene has been mutated or deleted.

A nucleic acid fragment encoding a "biologically active portion" of CARD-12 can be prepared by isolating a portion of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of  
15 ATCC \_\_\_\_\_, which encodes a polypeptide having a CARD-12 biological activity, expressing the encoded portion of CARD-12 protein (e.g., by recombinant expression in vitro) and assessing the activity of the encoded portion of CARD-12. For example, a nucleic acid fragment encoding a biologically active portion of CARD-12 includes a CARD domain, e.g., amino acids 1-88 of SEQ ID NO:2.

20 The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, and the cDNA of ATCC \_\_\_\_\_, due to degeneracy of the genetic code and thus encode the same CARD-12 protein as that encoded by the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC \_\_\_\_\_.

25 In addition to the CARD-12 nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, and the cDNA of ATCC \_\_\_\_\_, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of CARD-12 may exist within a population (e.g., the human population). Such genetic polymorphism in the CARD-12 gene may exist among individuals within a population  
30 due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a CARD-12 protein, preferably a mammalian CARD-12 protein. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of the CARD-12 gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in CARD-12  
35 that are the result of natural allelic variation and that do not alter the functional activity of CARD-12 are intended to be within the scope of the invention. Thus, e.g., 1%, 2%, 3%,

4%, or 5% of the amino acids in CARD-12 (e.g., 1, 2, 3, 4, 5, 6, 8, 10, 15, 20, or fewer amino acids) are replaced by another amino acid, preferably by conservative substitution.

Moreover, nucleic acid molecules encoding CARD-12 proteins from other species (CARD-12 orthologs/homologues), which have a nucleotide sequence which differs from that of a CARD-12 disclosed herein, are intended to be within the scope of the invention.

Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 150 (300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600, 1900, 2100, 2400, 2700, 3000, or 3100) nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence, preferably the coding sequence, of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC \_\_\_\_\_.

As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% (65%, 70%, preferably 75%) identical to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. An, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65° C (e.g., 50°C or 60°C or 65°C). Preferably, the isolated nucleic acid molecule of the invention that hybridizes under stringent conditions corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in a human cell in nature (e.g., encodes a natural protein).

In addition to naturally-occurring allelic variants of the CARD-12 sequence that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by mutation into the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC \_\_\_\_\_, thereby leading to changes in the amino acid sequence of the encoded protein without altering the functional ability of the protein. For example, one can make nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of CARD-12 protein without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the CARD-12, proteins of various species are predicted to be particularly unamenable to alteration.



For example, preferred CARD-12 proteins of the present invention contain at least one CARD domain. Additionally, a CARD-12 protein also contains at least one kinase-2 domain, at least one P-loop domain, at least one nucleotide binding site domain, and at least one LRR domain. Such conserved domains are less likely to be amenable to mutation. Other amino acid residues, however, (e.g., those that are not conserved or only semi-conserved among CARD-12 of various species) may not be essential for activity and thus are likely to be amenable to alteration.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding CARD-12 proteins that contain changes in amino acid residues that are not essential for activity. Such CARD-12 proteins differ in amino acid sequence from SEQ ID NO:2, and yet retain biological activity. In one embodiment, the isolated nucleic acid molecule includes a nucleotide sequence encoding a protein that includes an amino acid sequence that is at least about 45% identical, 65%, 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:2. An isolated nucleic acid molecule encoding a CARD-12 protein having a sequence which differs from that of SEQ ID NO:1, SEQ ID NO:3, or cDNA of ATCC \_\_\_\_\_, can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of CARD-12 (SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC \_\_\_\_\_) such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. Thus, for example, 1%, 2%, 3%, 5%, or 10% of the amino acids can be replaced by conservative substitution. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in CARD-12 is preferably replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of a CARD-12 coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for CARD-12 biological activity to identify mutants

that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

In an embodiment, a mutant CARD-12 protein can be assayed for: (1) the ability to form protein:protein interactions with proteins in the apoptotic signaling pathway; (2) the ability to bind a CARD-12 ligand; or (3) the ability to bind to an intracellular target protein.

The present invention encompasses antisense nucleic acid molecules, i.e., molecules which are complementary to a sense nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire CARD-12 coding strand, or to only a portion thereof, e.g., all or part of the protein coding region (or open reading frame). An antisense nucleic acid molecule can be antisense to a noncoding region of the coding strand of a nucleotide sequence encoding CARD-12. The noncoding regions ("5' and 3' untranslated regions") are the 5' and 3' sequences that flank the coding region and are not translated into amino acids. Given the coding strand sequences encoding CARD-12 disclosed herein, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of CARD-12 mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of CARD-12 mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of CARD-12 mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine,

2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated in situ such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a CARD-12 protein to thereby inhibit expression of the protein, e.g., by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An antisense nucleic acid molecule of the invention can be administered by direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

An antisense nucleic acid molecule of the invention can be an  $\alpha$ -anomeric nucleic acid molecule. An  $\alpha$ -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gaultier et al. (1987) *Nucleic Acids. Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue et al. (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue et al. (1987) *FEBS Lett.* 215:327-330).

The invention also encompasses ribozymes. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (e.g., hammerhead ribozymes (described in Haselhoff and Gerlach (1988) Nature 334:585-591)) can be used to catalytically cleave CARD-12 mRNA transcripts to thereby inhibit translation of CARD-12 mRNA. A ribozyme having specificity for a CARD-12-encoding nucleic acid can be designed based upon the nucleotide sequence of a CARD-12 cDNA disclosed herein. For example, a derivative of a Tetrahymena L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a CARD-12-encoding mRNA. See, e.g., Cech et al. U.S. Patent No. 4,987,071; and Cech et al. U.S. Patent No. 5,116,742. Alternatively, CARD-12 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, e.g., Bartel and Szostak (1993) Science 261:1411-1418.

The invention also encompasses nucleic acid molecules which form triple helical structures. For example, CARD-12 gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the CARD-12 (e.g., the CARD-12 promoter and/or enhancers) to form triple helical structures that prevent transcription of the CARD-12 gene in target cells. See generally, Helene (1991) Anticancer Drug Des. 6(6):569-84; Helene (1992) Ann. N.Y. Acad. Sci. 660:27-36; and Maher (1992) Bioassays 14(12):807-15.

In embodiments, the nucleic acid molecules of the invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup et al. (1996) Bioorganic & Medicinal Chemistry 4(1):5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, e.g., DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup et al. (1996) supra; Perry-O'Keefe et al. (1996) Proc. Natl. Acad. Sci. USA 93:14670-675.

PNAs of CARD-12 can be used for therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, e.g., inducing transcription or translation arrest or

inhibiting replication. PNAs of CARD-12 can also be used, e.g., in the analysis of single base pair mutations in a gene by, e.g., PNA directed PCR clamping; as artificial restriction enzymes when used in combination with other enzymes, e.g., S1 nucleases (Hyrup (1996) supra; or as probes or primers for DNA sequence and hybridization (Hyrup (1996) supra; Perry-O'Keefe et al. (1996) Proc. Natl. Acad. Sci. USA 93: 14670-675).

In another embodiment, PNAs of CARD-12 can be modified, e.g., to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of CARD-12 can be generated which may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, e.g., RNase H and DNA polymerases, to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup (1996) supra). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup (1996) supra and Finn et al. (1996) Nucleic Acids Research 24(17):3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, e.g., 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag et al. (1989) Nucleic Acid Res. 17:5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn et al. (1996) Nucleic Acids Research 24(17):3357-63). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser et al. (1975) Bioorganic Med. Chem. Lett. 5:1119-11124).

In other embodiments, the oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors in vivo), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al. (1989) Proc. Natl. Acad. Sci. USA 86:6553-6556; Lemaitre et al. (1987) Proc. Natl. Acad. Sci. USA 84:648-652; PCT Publication No. W0 88/09810) or the blood-brain barrier (see, e.g., PCT Publication No. W0 89/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (see, e.g., Krol et al. (1988) Bio/Techniques 6:958-976) or intercalating agents (see, e.g., Zon (1988) Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide,

hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

## **II. Isolated CARD-12 Proteins and Anti-CARD-12 Antibodies.**

5 One aspect of the invention pertains to isolated CARD-12 proteins, and biologically active portions thereof, as well as polypeptide fragments suitable for use as immunogens to raise anti-CARD-12 antibodies. In one embodiment, native CARD-12 proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, CARD-12  
10 proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a CARD-12 protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or  
15 tissue source from which the CARD-12 protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of CARD-12 protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, CARD-12 protein that is substantially free of  
20 cellular material includes preparations of CARD-12 protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of non-CARD-12 protein (also referred to herein as a "contaminating protein"). When the CARD-12 protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, 10%, or 5% of the volume  
25 of the protein preparation. When CARD-12 protein is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, i.e., it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. Accordingly such preparations of CARD-12 protein have less than about 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or non-CARD-12  
30 chemicals.

Biologically active portions of a CARD-12 protein include peptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the CARD-12 protein (e.g., the amino acid sequence shown in SEQ ID NO:2), which  
35 include less amino acids than the full length CARD-12 protein, and exhibit at least one activity of a CARD-12 protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the CARD-12 protein. A biologically active

portion of a CARD-12 protein can be a polypeptide which is, for example, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000 or more amino acids in length. Preferred biologically active polypeptides include one or more identified CARD-12 structural domains, e.g., the CARD domain (amino acids 1-88 of SEQ ID NO:2).

5        Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native CARD-12 protein.

10       CARD-12 protein has the amino acid sequence shown of SEQ ID NO:2. Other useful CARD-12 proteins are substantially identical to SEQ ID NO:2 and retain the functional activity of the protein of SEQ ID NO:2, yet differ in amino acid sequence due to natural allelic variation or mutagenesis.

15       A useful CARD-12 protein is a protein which includes an amino acid sequence at least about 45%, preferably 55%, 65%, 75%, 85%, 95%, or 99% identical to the amino acid sequence of SEQ ID NO:2, and retains the functional activity of the CARD-12 protein of SEQ ID NO:2.

20       To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical positions/total # of positions x 100).

25       The determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) Proc. Nat'l Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Nat'l Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences similar or homologous to CARD-12 nucleic acid molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in 35       Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402. When utilizing BLAST and

Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. When utilizing the ALIGN program for comparing nucleic acid sequences, a gap length penalty of 12, and a gap penalty of 4 can be used.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically exact matches are counted.

The invention also provides CARD-12 chimeric or fusion proteins. As used herein, a CARD-12 "chimeric protein" or "fusion protein" comprises a CARD-12 polypeptide operatively linked to a non-CARD-12 polypeptide. A "CARD-12 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to all or a portion (preferably a biologically active portion) of a CARD-12, whereas a "non-CARD-12 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially identical to the CARD-12 protein, e.g., a protein which is different from the CARD-12 proteins and which is derived from the same or a different organism. Within the fusion protein, the term "operatively linked" is intended to indicate that the CARD-12 polypeptide and the non-CARD-12 polypeptide are fused in-frame to each other. The heterologous polypeptide can be fused to the N-terminus or C-terminus of the CARD-12 polypeptide.

One useful fusion protein is a GST fusion protein in which the CARD-12 sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant CARD-12. In another embodiment, the fusion protein contains a signal sequence from another protein. In certain host cells (e.g., mammalian host cells), expression and/or secretion of CARD-12 can be increased through use of a heterologous signal sequence. For example, the gp67 secretory sequence of the baculovirus envelope protein can be used as a heterologous signal sequence (Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, 1992). Other examples of eukaryotic heterologous signal sequences include the secretory sequences of melittin and human placental alkaline phosphatase (Stratagene; La Jolla, California). In yet another example, useful prokaryotic heterologous signal sequences include the phoA secretory signal (Molecular cloning, Sambrook et al, second edition,



Cold spring harbor laboratory press, 1989) and the protein A secretory signal (Pharmacia Biotech; Piscataway, New Jersey).

In yet another embodiment, the fusion protein is a CARD-12-immunoglobulin fusion protein in which all or part of CARD-12 is fused to sequences derived from a member of the immunoglobulin protein family. The CARD-12-immunoglobulin fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject to inhibit an interaction between a CARD-12 ligand and a CARD-12 protein on the surface of a cell, to thereby suppress CARD-12-mediated signal transduction in vivo. The CARD-12-immunoglobulin fusion proteins can be used to affect the bioavailability of a CARD-12 cognate ligand. Inhibition of the CARD-12 ligand/CARD-12 interaction may be useful therapeutically for both the treatment of proliferative and differentiative disorders, as well as modulating (e.g., promoting or inhibiting) cell survival. Moreover, the CARD-12-immunoglobulin fusion proteins of the invention can be used as immunogens to produce anti-CARD-12 antibodies in a subject, to purify CARD-12 ligands and in screening assays to identify molecules which inhibit the interaction of CARD-12 with a CARD-12 ligand.

Preferably, a CARD-12 chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, e.g., Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST polypeptide). A CARD-12-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the CARD-12 protein.

The present invention also pertains to variants of the CARD-12 proteins which function as either CARD-12 agonists (mimetics) or as CARD-12 antagonists. Variants of the CARD-12 protein can be generated by mutagenesis, e.g., discrete point mutation or truncation of the CARD-12 protein. An agonist of the CARD-12 protein can retain

substantially the same, or a subset, of the biological activities of the naturally occurring form of the CARD-12 protein. An antagonist of the CARD-12 protein can inhibit one or more of the activities of the naturally occurring form of the CARD-12 protein by, for example, competitively binding to a downstream or upstream member of a cellular signaling cCARD-5ade which includes the CARD-12 protein. Thus, specific biological effects can be elicited by treatment with a variant of limited function. Treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein can have fewer side effects in a subject relative to treatment with the naturally occurring form of the CARD-12 proteins.

Variants of the CARD-12 protein which function as either CARD-12 agonists (mimetics) or as CARD-12 antagonists can be identified by screening combinatorial libraries of mutants, e.g., truncation mutants of the CARD-12 protein for CARD-12 protein agonist or antagonist activity. In one embodiment, a variegated library of CARD-12 variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of CARD-12 variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential CARD-12 sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of CARD-12 sequences therein. There are a variety of methods which can be used to produce libraries of potential CARD-12 variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential CARD-12 sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang (1983) Tetrahedron 39:3; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477).

Useful fragments of CARD-12, include fragments comprising or consisting of a domain or subdomain described herein, e.g., a kinase-2 domain or a CARD domain.

In addition, libraries of fragments of the CARD-12 protein coding sequence can be used to generate a variegated population of CARD-12 fragments for screening and subsequent selection of variants of a CARD-12 protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a CARD-12 coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA

to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal and internal fragments of various sizes of the CARD-12 protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of CARD-12 proteins. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify CARD-12 variants (Arkin and Yourvan (1992) Proc. Natl. Acad. Sci. USA 89:7811-7815; Delgrave et al. (1993) Protein Engineering 6(3):327-331).

An isolated CARD-12 protein, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind CARD-12 using standard techniques for polyclonal and monoclonal antibody preparation. The full-length CARD-12 protein can be used or, alternatively, the invention provides antigenic peptide fragments of CARD-12 for use as immunogens. The antigenic peptide of CARD-12 comprises at least 8 (preferably 10, 15, 20, or 30) amino acid residues of the amino acid sequence shown in SEQ ID NO:2 and encompasses an epitope of CARD-12 such that an antibody raised against the peptide forms a specific immune complex with CARD-12.

Useful antibodies include antibodies which bind to a domain or subdomain of CARD-12 described herein (e.g., a kinase-2 domain, a CARD domain, an NBS domain, a P-loop domain, or a LRR domain).

Preferred epitopes encompassed by the antigenic peptide are regions of CARD-12 that are located on the surface of the protein, e.g., hydrophilic regions. Other important criteria include a preference for a terminal sequence, high antigenic index (e.g., as predicted by Jameson-Wolf algorithm), ease of peptide synthesis (e.g., avoidance of prolines); and high surface probability (e.g., as predicted by the Emini algorithm; Figure 4).

A CARD-12 immunogen typically is used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed CARD-12 protein or a chemically synthesized CARD-12 polypeptide. The preparation  
5 can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic CARD-12 preparation induces a polyclonal anti-CARD-12 antibody response.

Accordingly, another aspect of the invention pertains to anti-CARD-12  
10 antibodies. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds an antigen, such as CARD-12. A molecule which specifically binds to CARD-12 is a molecule which binds CARD-12, but does not substantially bind other molecules in a sample, e.g., a biological sample, which  
15 naturally contains CARD-12. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')<sub>2</sub> fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind CARD-12. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of  
20 antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of CARD-12. A monoclonal antibody composition thus typically displays a single binding affinity for a particular CARD-12 protein with which it immunoreacts.

Polyclonal anti-CARD-12 antibodies can be prepared as described above by  
25 immunizing a suitable subject with a CARD-12 immunogen. The anti-CARD-12 antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized CARD-12. If desired, the antibody molecules directed against CARD-12 can be isolated from the mammal (e.g., from the blood) and further purified by  
30 well-known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the anti-CARD-12 antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) Nature 256:495-497, the human B  
35 cell hybridoma technique (Kozbor et al. (1983) Immunol Today 4:72), the EBV-hybridoma technique (Cole et al. (1985), Monoclonal Antibodies and Cancer

Therapy, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing various antibodies monoclonal antibody hybridomas is well known (see generally Current Protocols in Immunology (1994) Coligan et al. (eds.) John Wiley & Sons, Inc., New York, NY). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a CARD-12 immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds CARD-12.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-CARD-12 monoclonal antibody (see, e.g., Current Protocols in Immunology, supra; Galfre et al. (1977) Nature 266:55052; R.H. Kenneth, in Monoclonal Antibodies: A New Dimension In Biological Analyses, Plenum Publishing Corp., New York, New York (1980); and Lerner (1981) Yale J. Biol. Med., 54:387-402). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line, e.g., a myeloma cell line that is sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, e.g., the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind CARD-12, e.g., using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-CARD-12 antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with CARD-12 to thereby isolate immunoglobulin library members that bind CARD-12. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia Recombinant Phage Antibody System, Catalog No.

27-9400-01; and the Stratagene SurfZAP Phage Display Kit, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No. WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J.* 12:725-734.

Additionally, recombinant anti-CARD-12 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better et al. (1988) *Science* 240:1041-1043; Liu et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu et al. (1987) *J. Immunol.* 139:3521-3526; Sun et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura et al. (1987) *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al. (1988) *J. Natl. Cancer Inst.* 80:1553-1559; Morrison, (1985) *Science* 229:1202-1207; Oi et al. (1986) *Bio/Techniques* 4:214; U.S. Patent 5,225,539; Jones et al. (1986) *Nature* 321:552-525; Verhoeyan et al. (1988) *Science* 239:1534; and Beidler et al. (1988) *J. Immunol.* 141:4053-4060.

An anti-CARD-12 antibody (e.g., monoclonal antibody) can be used to isolate CARD-12 by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-CARD-12 antibody can facilitate the purification of natural CARD-12 from cells and of recombinantly produced CARD-12 expressed in host cells. Moreover, an anti-CARD-12 antibody can be used to detect CARD-12 protein (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the CARD-12 protein. Anti-CARD-12 antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, e.g., to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials,

luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include  
5 umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ .

Further, an antibody (or fragment thereof) may be conjugated to a therapeutic  
10 moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone,  
15 glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan,  
20 dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological  
25 response. The drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as  
30 tumor necrosis factor,  $\alpha$ -interferon,  $\beta$ -interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), or other growth factors.

35 Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Arnon et al., "Monoclonal Antibodies for Immunotargeting of Drugs in Cancer

Therapy", in *Monoclonal Antibodies and Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies for Drug Delivery", in *Controlled Drug Delivery* (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers of Cytotoxic Agents in Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological and Clinical Applications*, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, and Future Prospective of The Therapeutic Use of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies for Cancer Detection and Therapy*, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation and Cytotoxic Properties of Antibody-Toxin Conjugates", *Immunol. Rev.*, 62:119-58 (1982). Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

In addition, antibodies of the invention, either conjugated or not conjugated to a therapeutic moiety, can be administered together or in combination with a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. The order of administration of the antibody and therapeutic moiety can vary. For example, in some embodiments, the antibody is administered concurrently (through the same or different delivery devices, e.g., syringes) with the therapeutic moiety. Alternatively, the antibody can be administered separately and prior to the therapeutic moiety. Still alternatively, the therapeutic moiety is administered separately and prior to the antibody. In many embodiments, these administration regimens will be continued for days, months or years.

Another aspect of the invention relates to a method for inducing an immunological response in a mammal which comprises inoculating the mammal with a CARD-12 polypeptide, adequate to produce antibody and/or T cell immune response to protect the animal from the diseases hereinbefore mentioned, amongst others. Yet another aspect of the invention relates to a method of inducing immunological response in a mammal which comprises, delivering a CARD-12 polypeptide via a vector directing expression of the polynucleotide and coding for the polypeptide in vivo in order to induce such an immunological response to produce antibody to protect the animal from diseases.

A further aspect of the invention relates to an immunological/vaccine formulation (composition) which, when introduced into a mammalian host, induces an immunological response in that mammal to a CARD-12 polypeptide of the present invention wherein the composition comprises a polypeptide or polynucleotide of CARD-12. The vaccine formulation may further comprise a suitable carrier. Since a polypeptide may be broken down in the stomach, it is preferably administered parenterally (for instance, subcutaneous, intramuscular, intravenous, or intradermal injection). Formulations



suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation instonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

### III. Computer Readable Means

The nucleotide or amino acid sequences of the invention are also provided in a variety of mediums to facilitate use thereof. As used herein, "provided" refers to a manufacture, other than an isolated nucleic acid or amino acid molecule, which contains a nucleotide or amino acid sequence of the present invention. Such a manufacture provides the nucleotide or amino acid sequences, or a subset thereof (e.g., a subset of open reading frames (ORFs)) in a form which allows a skilled artisan to examine the manufacture using means not directly applicable to examining the nucleotide or amino acid sequences, or a subset thereof, as they exist in nature or in purified form.

In one application of this embodiment, a nucleotide or amino acid sequence of the present invention can be recorded on computer readable media. As used herein, "computer readable media" refers to any medium that can be read and accessed directly by a computer. Such media include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media. This skilled artisan will readily appreciate how any of the presently known computer readable mediums can be used to create a manufacture comprising computer readable medium having recorded thereon a nucleotide or amino acid sequence of the present invention.

As used herein, "recorded" refers to a process for storing information on computer readable medium. The skilled artisan can readily adopt any of the presently known methods for recording information on computer readable medium to generate manufactures comprising the nucleotide or amino acid sequence information of the present invention.

A variety of data storage structures are available to a skilled artisan for creating a computer readable medium having recorded thereon a nucleotide or amino acid sequence of the present invention. The choice of the data storage structure will generally be based on the means chosen to access the stored information. In addition, a variety of data processor programs and formats can be used to store the nucleotide sequence information of the present invention on computer readable medium. The sequence information can be represented in a work processing test file, formatted in commercially-available software such as WordPerfect and Microsoft Word, or represented in the form of an CARD-5II file, stored in a database application, such as DB2, Sybase, Oracle, or the like. The skilled artisan can readily adapt any number of data processor structuring formats (e.g., text file or database) in order to obtain computer readable medium having recorded thereon the nucleotide sequence information of the present invention.

By providing the nucleotide or amino acid sequences of the invention in computer readable form, the skilled artisan can routinely access the sequence information for a variety of purposes. For example, one skilled in the art can use the nucleotide or amino acid sequences of the invention in computer readable form to compare a target sequence or a target structural motif with the sequence information stored within the data storage means. Search means are used to identify fragments or regions of the sequences of the invention which match a particular target sequence or target motif.

As used herein, a "target sequence" can be any DNA or amino acid sequence of six or more nucleotides or two or more amino acids. A skilled artisan can readily recognize that the longer a target sequence is, the less likely a target sequence will be present as a random occurrence in the database. The most preferred sequence length of a target sequence is from about 10 to 100 amino acids or from about 30 to 300 nucleotide residues. However, it is well recognized that commercially important fragments, such as sequence fragments involved in gene expression and protein processing, may be of shorter length.

As used herein, "a target structural motif," or "target motif," refers to any rationally selected sequence or combination of sequences in which the sequence(s) are chosen based on a three-dimensional configuration formed upon the folding of the target motif. There are a variety of target motifs known in the art. Protein target motifs include, but are not limited to, enzyme active sites and signal sequences. Nucleic acid target motifs include, but are not limited to, promoter sequences, hairpin structures and inducible expression elements (protein binding sequences).

Computer software is publicly available which allows a skilled artisan to access sequence information provided in a computer readable medium for analysis and

comparison to other sequences. A variety of know algorithms are disclosed publicly and a variety of commercially available software for conducting search means are and can be used in the computer-based systems of the present invention. Examples of such software include, but is not limited to, MacPattern (EMBL), BLASTIN and BLASTX (NCBIA).

5 For example, software that implements the BLAST (*Altschul et al. (1990) J. of Mol. Biol. 215:403-410*) and BLAZE (*Brutlag et al. (1993) Comp. Chem. 17:203-207*) search algorithms on a Sybase system can be used to identify open reading frames (ORFs) of the sequences of the invention which contain homology to ORFs or proteins from other libraries. Such ORFs are protein-encoding fragments and are useful in  
10 producing commercially important proteins such as enzymes used in various reactions and in the production of commercially useful metabolites.

#### IV. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, preferably expression vectors,  
15 containing a nucleic acid encoding CARD-12 (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated  
20 into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain  
25 vectors, expression vectors, are capable of directing the expression of genes to which they are operatively linked. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids (vectors). However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which  
30 serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively  
35 linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is

linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in  
5 Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific  
10 regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g.,  
15 CARD-12 proteins, mutant forms of CARD-12, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of CARD-12 in prokaryotic or eukaryotic cells, e.g., bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, Gene Expression Technology:  
20 Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either  
25 fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion  
30 expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and  
35 Johnson (1988) Gene 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5

(Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 *gn10*-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 *gn1*). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident  $\phi$  prophage harboring a T7 *gn1* gene under the transcriptional control of the lacUV5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a bacterial having an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the CARD-12 expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari et al. (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al. (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), pGBT9 (Clontech, Palo Alto, CA), pGAD10 (Clontech, Palo Alto, CA), pYADE4 and pYGAE2 and pYPGE2 (Brunelli and Pall, (1993) *Yeast* 9:1299-1308), pYPGE15 (Brunelli and Pall, (1993) *Yeast* 9:1309-1318), pACTII (Dr. S.E. Elledge, Baylor College of Medicine), and picZ (Invitrogen Corp, San Diego, CA).

Alternatively, CARD-12 can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf9 cells) include the pAc series (Smith et al. (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed (1987) *Nature* 329:840), pCI (Promega), and pMT2PC (Kaufman et al. (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements.

For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook et al. (supra).

5 In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert et al. (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji et al. (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund et al. (1985) *Science* 230:912-916),  
10 and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166).  
Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the  $\alpha$ -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

20 The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to CARD-12 mRNA. Regulatory sequences operatively  
25 linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or  
30 attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub et al. (Reviews - Trends in Genetics, Vol. 1(1) 1986).

35 Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention or isolated nucleic acid molecule of the invention has

been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, CARD-12 protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA or an isolated nucleic acid molecule of the invention can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (*supra*), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In some cases vector DNA is retained by the host cell. In other cases the host cell does not retain vector DNA and retains only an isolated nucleic acid molecule of the invention carried by the vector. In some cases, and isolated nucleic acid molecule of the invention is used to transform a cell without the use of a vector.

In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding CARD-12 or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (i.e., express) a CARD-12 protein. Accordingly, the invention further provides methods for producing CARD-12 protein using the host cells

of the invention. In one embodiment, the method comprises culturing the host cell of the invention (into which a recombinant expression vector or isolated nucleic acid molecule encoding CARD-12 has been introduced) in a suitable medium such that CARD-12 protein is produced. In another embodiment, the method further comprises isolating  
5 CARD-12 from the medium or the host cell.

CARD-12 nucleic acid molecules can be used in viral gene delivery systems for gene therapy, e.g., adenoviral or retroviral gene delivery systems.

CARD-12 nucleic acid molecules can also be used in non-viral gene delivery systems for gene therapy. Thus, another aspect of the invention pertains to non-viral  
10 gene delivery systems, such as plasmid-based gene delivery systems. Non-viral gene delivery systems are described in detail by Huang et al. ((1999) Nonviral Vectors for Gene Therapy, Academic Press, San Diego, CA). Nonviral vectors have several potential advantages over their viral counterparts, including: reduced immunogenicity; low acute toxicity; simplicity; and ease of large scale production. Nonviral vectors can be delivered  
15 as naked DNA, by bioballistic bombardment, and in various complexes, including liposome/DNA complexes (lipoplexes), polymer/DNA complexes (polyplexes), and liposome/polymer/DNA complexes (lipopolyplexes). Nonviral vectors may be administered by various routes, e.g., intravenous injection, peritoneal injection, intramuscular injection, subcutaneous injection, intratracheal injection, and  
20 aerosolization.

Naked DNA (i.e. free from association with, e.g., transfection-facilitating proteins, viral particles, liposomal formulations, charged lipids and calcium phosphate precipitating), can be expressed at its injection site or at a remote site. For example, naked DNA can be injected directly into skeletal muscle, liver, heart muscle, and tumor  
25 tissue. For systemic administration, plasmid DNA may need to be protected from degradation by endonucleases during delivery from the site of administration to the site of gene expression.

Bioballistic bombardment, also known as gene gun, allows for the penetration of target cells *in vitro*, *ex vivo*, or *in vivo*. In this technique, DNA-coated gold particles are  
30 accelerated to a high velocity by an electric arc generated by a high voltage discharge. The method is effective for a variety of organ types, including skin, liver, muscle, spleen, and pancreas. The gene gun transfer method is not dependent upon specific cell surface receptors, cell cycle status, or the size of the DNA vector. Useful gene gun devices include the Accell® (PowderJect Vaccines, Inc.) and the Helios™ (Bio-Rad). These  
35 devices create a compressed shock wave of helium gas, accelerating DNA-coated gold



(or tungsten) particles to high speed, whereby the particles have sufficient momentum to penetrate a target tissue.

Lipoplexes are typically made up of three components: a cationic lipid, a neutral colipid, and plasmid DNA that encodes one or more genes of interest. Commonly used cationic lipids include DOTMA, DMRIE, DC-chol, DOTAP, DMRIE, DDAB, DODAB/C, DOGS, DOSPA, SAINT-n, DOSPER, DPPES, DORIE, GAP-DLRIE, and DOTIM. Dioleoyl (DO) and dimyristoyl (DM) chains are thought to be especially effective for gene delivery. Cationic lipids are typically composed of a positively charged headgroup, a hydrophobic lipid anchor, and a linker that connects the headgroup and anchor. Cationic lipids used in lipoplexes can be divided into two broad classes: those that use cholesterol as the lipid anchor and those that use diacyl chains of varying lengths and extent of saturation. The number of protonatable amines on the headgroup may affect transfection activity, with multivalent headgroups being generally more active than monovalent headgroups. The linker can be made of a variety of chemical structures, e.g., ether, amide, carbamate, amine, urea, ester, and peptide bonds. Neutral colipids of lipoplexes commonly include DOPE, DOPC, and cholesterol. Generally, DOPE is used as the neutral colipid with cationic lipids that are based on cholesterol (e.g., DC-chol, GL-67) and cholesterol is used as the neutral colipid with cationic lipids that harbor diacyl chains as the hydrophobic anchor (e.g., DOTAP, DOTIM).

Polyplexes are formed when cationic polymers are mixed with DNA. Cationic polymers used to form polyplexes are of two general types: linear polymers such as polylysine and spermine; and the branched chain, spherical, or globular polycations such as polyethyleneimine and dendrimers. Lipopolyplexes are formed by the incorporation of polylysine into a lipoplex to form ternary complexes. DNA can be complexed with a natural biopolymer, e.g., gelatin or chitosan, functioning as a gene carrier to form nanospheres. Such biodegradable nanospheres have several advantages, including the coencapsulation of bioactive agents, e.g. nucleic acids and drugs, and the sustained release of the DNA. Gelatin-DNA or chitosan-DNA nanospheres are synthesized by mixing the DNA solution with an aqueous solution of gelatin or chitosan.

The effectiveness nonviral vectors may be enhanced by conjugation to ligands that direct the vector either to a particular cell type or to a particular location within a cell. Antibodies and other site-specific proteins can be attached to a vector, e.g., on the surface of the vector or incorporated in the membrane. Following injection, these vectors bind efficiently and specifically to a target site. With respect to liposomes, ligands to a cell surface receptor can be incorporated into the surface of a liposome by covalently modifying the ligand with a lipid group and adding it during the formation of liposomes.

The following classes of ligands can be incorporated into the nonviral DNA delivery complexes of the invention in order to make them more effective for gene delivery: (1) peptides, e.g., peptides having a specific cell surface receptor so that complexes will be targeted to specific cells bearing the receptor; (2) nuclear localization signals, e.g., to promote efficient entry of DNA into the nucleus; (3) pH-sensitive ligands, to encourage endosomal escape; (4) steric stabilizing agents, to prevent destabilization of the complexes after introduction into the biological milieu. Gene chemistry approaches, e.g. peptide nucleic acids, can be used to couple ligands to DNA to improve the *in vivo* bioavailability and expression of the DNA.

In plasmid-based, non-viral gene delivery systems it is often useful to link a polypeptide (e.g., an antibody), nucleic acid molecule, or other compound to the gene delivery plasmid such that the polypeptide, nucleic acid molecule or other compound remains associated with the plasmid following intracellular delivery in a manner that does not interfere with the transcriptional activity of the plasmid. This can be accomplished using an appropriate biotin-conjugated peptide nucleic acid (PNA) clamp. A sequence complementary to the biotin-conjugated PNA clamp is inserted into the gene delivery plasmid. The biotin-conjugated PNA will bind essentially irreversibly to the complementary sequence inserted into the plasmid. A polypeptide, nucleic acid molecule or other compound of interest can be conjugated to streptavidin. The streptavidin conjugate can bind to the biotin-PNA clamp bound to the plasmid. In this manner, a polypeptide, nucleic acid molecule or other compound can be bound to a gene delivery plasmid such that the polypeptide, nucleic acid molecule or other compound remains bound to the plasmid even within a cell. Importantly, the PNA clamp-binding site in the plasmid must be chosen so as not to interfere with a needed promoter/enhancer or coding region or otherwise disrupt the expression of the gene in the plasmid. An alternative approach employs a maleimide-conjugated PNA clamp. Polypeptides, nucleic acid molecules and other compounds containing a free thiol residue may be conjugated directly to the maleimide-PNA-DNA hybrid. As with the biotin-conjugated method, this conjugation does not disturb the transcriptional activity of the plasmid if the PNA-binding site is chosen to be in a region of the plasmid not essential for gene activity. Both of these approaches are described in detail by Zelphati et al. ((2000) *BioTechniques* 28:304-315).

The host cells of the invention can also be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which CARD-12-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in

which exogenous CARD-12 sequences have been introduced into their genome or homologous recombinant animals in which endogenous CARD-12 sequences have been altered. Such animals are useful for studying the function and/or activity of CARD-12 and for identifying and/or evaluating modulators of CARD-12 activity. As used herein, a  
5 "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which  
10 remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, an "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous CARD-12 gene has been altered by homologous recombination between the endogenous gene and an exogenous  
15 DNA molecule introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing CARD-12-encoding nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a  
20 pseudopregnant female foster animal. The CARD-12 cDNA sequence, e.g., that of SEQ ID NO:1, SEQ ID NO:3 or the cDNA of ATCC \_\_\_\_ can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homolog or ortholog of the human CARD-12 gene, such as a mouse CARD-12 gene, can be isolated based on hybridization to the human CARD-12 cDNA and used as a transgene. Intronic sequences  
25 and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the CARD-12 transgene to direct expression of CARD-12 protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art  
30 and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, U.S. Patent No. 4,873,191 and in Hogan, Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified  
35 based upon the presence of the CARD-12 transgene in its genome and/or expression of CARD-12 mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic

animals carrying a transgene encoding CARD-12 can further be bred to other transgenic animals carrying other transgenes.

To create an homologous recombinant animal, a vector is prepared which contains at least a portion of a CARD-12 gene (e.g., a human or a non-human homolog of the CARD-12 gene, e.g., a murine CARD-12 gene) into which a deletion, addition or substitution has been introduced to thereby alter, e.g., functionally disrupt, the CARD-12 gene. In an embodiment, the vector is designed such that, upon homologous recombination, the endogenous CARD-12 gene is functionally disrupted (i.e., no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous CARD-12 gene is mutated or otherwise altered but still encodes functional protein (e.g., the upstream regulatory region can be altered to thereby alter the expression of the endogenous CARD-12 protein). In the homologous recombination vector, the altered portion of the CARD-12 gene is flanked at its 5' and 3' ends by additional nucleic acid of the CARD-12 gene to allow for homologous recombination to occur between the exogenous CARD-12 gene carried by the vector and an endogenous CARD-12 gene in an embryonic stem cell. The additional flanking CARD-12 nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see, e.g., Thomas and Capecchi (1987) Cell 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced CARD-12 gene has homologously recombined with the endogenous CARD-12 gene are selected (see, e.g., Li et al. (1992) Cell 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see, e.g., Bradley in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley (1991) Current Opinion in Bio/Technology 2:823-829 and in PCT Publication Nos. WO 90/11354, WO 91/01140, WO 92/0968, and WO 93/04169.

In another embodiment, transgenic non-humans animals can be produced which contain selected systems which allow for regulated expression of the transgene. One

example of such a system is the cre/loxP recombinase system of bacteriophage P1. For a description of the cre/loxP recombinase system, see, e.g., Lakso et al. (1992) Proc. Natl. Acad. Sci. USA 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al. (1991) Science 251:1351-1355. If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut et al. (1997) Nature 385:810-813 and PCT Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, e.g., a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter Go phase. The quiescent cell can then be fused, e.g., through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, e.g., the somatic cell, is isolated.

## V. Pharmaceutical Compositions

The CARD-12 nucleic acid molecules, CARD-12 proteins, and anti-CARD-12 antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The invention includes methods for preparing pharmaceutical compositions for modulating the expression or activity of a polypeptide or nucleic acid of the invention.

Such methods comprise formulating a pharmaceutically acceptable carrier with an agent which modulates expression or activity of a polypeptide or nucleic acid of the invention. Such compositions can further include additional active agents. Thus, the invention further includes methods for preparing a pharmaceutical composition by formulating a pharmaceutically acceptable carrier with an agent which modulates expression or activity of a polypeptide or nucleic acid of the invention and one or more additional active compounds.

The agent which modulates expression or activity may, for example, be a small molecule. For example, such small molecules include peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. It is understood that appropriate doses of small molecule agents depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention. Exemplary doses include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. Such appropriate doses may be determined using the assays described herein. When one or more of these small molecules is to be administered to an animal (e.g., a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular subject will depend upon a variety of factors including the activity of the specific compound

employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

5 A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils,  
10 polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as CARD-Sorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be  
15 adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the  
20 extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be  
25 preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required  
30 particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, CARD-Sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged  
35 absorption of the injectable compositions can be brought about by including in the

composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

5 Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a CARD-12 protein or anti-CARD-12 antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

15 Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or 25 saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

30 Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in 35 the art.



The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

For antibodies, the preferred dosage is 0.1 mg/kg to 100 mg/kg of body weight (generally 10 mg/kg to 20 mg/kg). If the antibody is to act in the brain, a dosage of 50 mg/kg to 100 mg/kg is usually appropriate. Generally, partially human antibodies and fully human antibodies have a longer half-life within the human body than other antibodies. Accordingly, lower dosages and less frequent administration is often possible. Modifications such as lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration (e.g., into the brain). A method for lipidation of antibodies is described by Cruikshank et al. ((1997) J. Acquired Immune Deficiency Syndromes and Human Retrovirology 14:193).

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Patent 5,328,470) or by stereotactic injection (see, e.g., Chen et al. (1994) Proc. Natl. Acad. Sci. USA

91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The gene therapy vectors of the invention can be either viral or non-viral. Examples of plasmid-based, non-viral vectors are discussed in Huang et al. (1999) Nonviral Vectors for Gene Therapy (supra). A modified plasmid is one example of a non-viral gene delivery system. Peptides, proteins (including antibodies), and oligonucleotides may be stably conjugated to plasmid DNA by methods that do not interfere with the transcriptional activity of the plasmid (Zelphati et al. (2000) BioTechniques 28:304-315). The attachment of proteins and/or oligonucleotides may influence the delivery and trafficking of the plasmid and thus render it a more effective pharmaceutical composition.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

## **VI. Uses and Methods of the Invention**

The nucleic acid molecules, proteins, protein homologues, and antibodies described herein can be used in one or more of the following methods: a) screening assays; b) detection assays (e.g., chromosomal mapping, tissue typing, forensic biology), c) predictive medicine (e.g., diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenomics); and d) methods of treatment (e.g., therapeutic and prophylactic). A CARD-12 protein interacts with other cellular proteins and can thus be used for (i) regulation of cellular proliferation; (ii) regulation of cellular differentiation; and (iii) regulation of cell survival. The isolated nucleic acid molecules of the invention can be used to express CARD-12 protein (e.g., via a recombinant expression vector in a host cell in gene therapy applications), to detect CARD-12 mRNA (e.g., in a biological sample) or a genetic lesion in a CARD-12 gene, and to modulate CARD-12 activity. In addition, the CARD-12 proteins can be used to screen drugs or compounds which modulate the CARD-12 activity or expression as well as to treat disorders characterized by insufficient or excessive production of CARD-12 protein or production of CARD-12 protein forms which have decreased or aberrant activity compared to CARD-12 wild type protein. In addition, the anti-CARD-12 antibodies of the invention can be used to detect and isolate CARD-12 proteins and modulate CARD-12 activity.

This invention further pertains to novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

#### A. Screening Assays

5 The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, i.e., candidate or test compounds or agents (e.g., peptides, peptidomimetics, small molecules or other drugs) which bind to CARD-12 proteins or biologically active portions thereof or have a stimulatory or inhibitory effect on, for example, CARD-12 expression or CARD-12 activity. An example of a biologically  
10 active portion of human CARD-12 is amino acids 139-227 encoding a CARD domain.

Among the screening assays provided by the invention are screening to identify molecules that prevent the dimerization of CARD-12, screening to identify molecules which block the binding of a CARD containing polypeptide to CARD-12, and screening to identify a competitive inhibitor of the binding of a nucleotide to the nucleotide binding  
15 site of CARD-12. Screening assays, e.g., dimerization assays, can employ full-length CARD-12 or a portion of CARD-12, e.g. the CARD domain, the nucleotide binding site domain, or the NAIP homology domain.

In one embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of a CARD-12 proteins or  
20 polypeptides or biologically active portions thereof. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using  
25 affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) *Anticancer Drug Des.* 12:145). Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann et al. (1994). *J. Med. Chem.* 37:2678;  
30 Cho et al. (1993) *Science* 261:1303; Carrell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carrell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop et al. (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992)  
35 *Bio/Techniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos.

5,571,698; 5,403,484; and 5,223,409), plasmids (Cull et al. (1992) Proc. Natl. Acad. Sci. USA 89:1865-1869) or on phage (Scott and Smith (1990) Science 249:386-390; Devlin (1990) Science 249:404-406; Cwirla et al. (1990) Proc. Natl. Acad. Sci. 87:6378-6382; and Felici (1991) J. Mol. Biol. 222:301-310).

5        Determining the ability of the test compound to modulate the activity of CARD-12 or a biologically active portion thereof can be accomplished, for example, by determining the ability of the CARD-12 protein to bind to or interact with a CARD-12 target molecule. As used herein, a "target molecule" is a molecule with which a CARD-12 protein binds or interacts in nature, for example, a molecule associated with the  
10        internal surface of a cell membrane or a cytoplasmic molecule. A CARD-12 target molecule can be a non-CARD-12 molecule or a CARD-12 protein or polypeptide of the present invention. In one embodiment, a CARD-12 target molecule is a component of an apoptotic signal transduction pathway. The target, for example, can be a second intracellular protein which has catalytic activity or a protein which facilitates the  
15        association of downstream signaling molecules with CARD-12.

      Determining the ability of the test compound to modulate the activity of CARD-12 or a biologically active portion thereof can be accomplished, for example, by determining the ability of the CARD-12 protein to bind to or interact with any of the specific proteins listed in the previous paragraph as CARD-12 target molecules. In  
20        another embodiment, CARD-12 target molecules include all proteins that bind to a CARD-12 protein or a fragment thereof in a two-hybrid system binding assay which can be used without undue experimentation to isolate such proteins from cDNA or genomic two-hybrid system libraries. The binding assays described in this section can be cell-based or cell free (described subsequently).

25        Determining the ability of the CARD-12 protein to bind to or interact with a CARD-12 target molecule can be accomplished by one of the methods described above for determining direct binding. In an embodiment, determining the ability of the CARD-12 protein to bind to or interact with a CARD-12 target molecule can be accomplished by determining the activity of the target molecule. For example, the activity of the target  
30        molecule can be determined by detecting induction of a cellular second messenger of the target (e.g., intracellular Ca<sup>2+</sup>, diacylglycerol, IP<sub>3</sub>, etc.), detecting catalytic/enzymatic activity of the target on an appropriate substrate, detecting the induction of a reporter gene (e.g., a CARD-12-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, e.g. luciferase), or detecting a cellular response, for  
35        example, cell survival, cellular differentiation, or cell proliferation. In addition, and in another embodiment, genes induced by CARD-12 expression can be identified by

expressing CARD-12 in a cell line and conducting a transcriptional profiling experiment wherein the mRNA expression patterns of the cell line transformed with an empty expression vector and the cell line transformed with a CARD-12 expression vector are compared. The promoters of genes induced by CARD-12 expression can be operatively  
5 linked to reporter genes suitable for screening such as luciferase, secreted alkaline phosphatase, or beta-galactosidase and the resulting constructs could be introduced into appropriate expression vectors. A recombinant cell line containing CARD-12 and transfected with an expression vector containing a CARD-12 responsive promoter operatively linked to a reporter gene can be used to identify test compounds that  
10 modulate CARD-12 activity by assaying the expression of the reporter gene in response to contacting the recombinant cell line with test compounds. CARD-12 agonists can be identified as increasing the expression of the reporter gene and CARD-12 antagonists can be identified as decreasing the expression of the reporter gene.

In another embodiment of the invention, the ability of a test compound to  
15 modulate the activity of CARD-12, or biologically active portions thereof can be determined by assaying the ability of the test compound to modulate CARD-12-dependent pathways or processes where the CARD-12 target proteins that mediate the CARD-12 effect are known or unknown. Potential CARD-12-dependent pathways or processes include, but are not limited to, the modulation of cellular signal transduction  
20 pathways and their related second messenger molecules (e.g., intracellular Ca<sup>2+</sup>, diacylglycerol, IP<sub>3</sub>, cAMP etc.), cellular enzymatic activities, cellular responses (e.g., cell survival, cellular differentiation, or cell proliferation), or the induction or repression of cellular or heterologous mRNAs or proteins. CARD-12-dependent pathways or processes could be assayed by standard cell-based or cell free assays appropriate for the  
25 specific pathway or process under study.

In yet another embodiment, an assay of the present invention is a cell-free assay comprising contacting a CARD-12 protein or biologically active portion thereof with a test compound and determining the ability of the test compound to bind to the CARD-12 protein or biologically active portion thereof. Binding of the test compound to the  
30 CARD-12 protein can be determined either directly or indirectly as described above. In one embodiment, a competitive binding assay includes contacting the CARD-12 protein or biologically active portion thereof with a compound known to bind CARD-12 to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a CARD-12 protein, wherein determining the  
35 ability of the test compound to interact with a CARD-12 protein comprises determining

the ability of the test compound to preferentially bind to CARD-12 or biologically active portion thereof as compared to the known binding compound.

In another embodiment, an assay is a cell-free assay comprising contacting CARD-12 protein or biologically active portion thereof with a test compound and  
5 determining the ability of the test compound to modulate (e.g., stimulate or inhibit) the activity of the CARD-12 protein or biologically active portion thereof. Determining the ability of the test compound to modulate the activity of CARD-12 can be accomplished, for example, by determining the ability of the CARD-12 protein to bind to a CARD-12 target molecule by one of the methods described above for determining direct binding. In  
10 an alternative embodiment, determining the ability of the test compound to modulate the activity of CARD-12 can be accomplished by determining the ability of the CARD-12 protein to further modulate a CARD-12 target molecule. For example, the catalytic/enzymatic activity of the target molecule on an appropriate substrate can be determined as previously described.

In yet another embodiment, the cell-free assay comprises contacting the CARD-12 protein or biologically active portion thereof with a known compound which binds CARD-12 to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a CARD-12 protein, wherein determining the ability of the test compound to interact with a CARD-12 protein  
20 comprises determining the ability of the CARD-12 protein to preferentially bind to or modulate the activity of a CARD-12 target molecule. The cell-free assays of the present invention are amenable to use of either the soluble form or a membrane-associated form of CARD-12. A membrane-associated form of CARD-12 refers to CARD-12 that interacts with a membrane-bound target molecule. In the case of cell-free assays  
25 comprising the membrane-associated form of CARD-12, it may be desirable to utilize a solubilizing agent such that the membrane-associated form of CARD-12 is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®,  
30 Isotridecypoly(ethylene glycol ether)<sub>n</sub>, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In more than one embodiment of the above assay methods of the present  
35 invention, it may be desirable to immobilize either CARD-12 or its target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the

proteins, as well as to accommodate automation of the assay. Binding of a test compound to CARD-12, or interaction of CARD-12 with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/CARD-12 fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical; St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or CARD-12 protein, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of CARD-12 binding or activity determined using standard techniques. In an alternative embodiment, MYC or HA epitope tag CARD-12 fusion proteins or MYC or HA epitope tag target fusion proteins can be adsorbed onto anti-MYC or anti-HA antibody coated microbeads or onto anti-MYC or anti-HA antibody coated microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or CARD-12 protein; and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of CARD-12 binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, CARD-12 or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated CARD-12 target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals; Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with CARD-12 or target molecules but which do not interfere with binding of the protein to its target molecule can be derivatized to the wells

of the plate, and unbound target or protein trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes and epitope tag immobilized complexes, include immunodetection of complexes using antibodies reactive with the CARD-12 or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the CARD-12 or a target molecule.

In another embodiment, modulators of CARD-12 expression are identified in a method in which a cell is contacted with a candidate compound and the expression of the CARD-12 promoter, mRNA or protein in the cell is determined. The level of expression of CARD-12 mRNA or protein in the presence of the candidate compound is compared to the level of expression of CARD-12 mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of CARD-12 expression based on this comparison. For example, when expression of CARD-12 mRNA or protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of CARD-12 mRNA or protein expression. Alternatively, when expression of CARD-12 mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of CARD-12 mRNA or protein expression. The level of CARD-12 mRNA or protein expression in the cells can be determined by methods described herein for detecting CARD-12 mRNA or protein. The activity of the CARD-12 promoter can be assayed by linking the CARD-12 promoter to a reporter gene such as luciferase, secreted alkaline phosphatase, or beta-galactosidase and introducing the resulting construct into an appropriate vector, transfecting a host cell line, and measuring the activity of the reporter gene in response to test compounds.

In yet another aspect of the invention, the CARD-12 proteins can be used as "bait proteins" in a two-hybrid assay or three hybrid assay (see, e.g., U.S. Patent No. 5,283,317; Zervos et al. (1993) *Cell* 72:223-232; Madura et al. (1993) *J. Biol. Chem.* 268:12046-12054; Bartel et al. (1993) *Bio/Techniques* 14:920-924; Iwabuchi et al. (1993) *Oncogene* 8:1693-1696; and PCT Publication No. WO 94/10300), to identify other proteins, which bind to or interact with CARD-12 ("CARD-12-binding proteins" or "CARD-12-bp") and modulate CARD-12 activity. Such CARD-12-binding proteins are also likely to be involved in the propagation of signals by the CARD-12 proteins as, for example, upstream or downstream elements of the CARD-12 pathway.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the



assay utilizes two different DNA constructs. In one construct, the gene that codes for CARD-12 is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene  
5 that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, in vivo, forming a CARD-12-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor.  
10 Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with CARD-12.

In an embodiment of the invention, the ability of a test compound to modulate the activity of CARD-12, or a biologically active portion thereof can be determined by  
15 assaying the ability of the test compound to block the binding of CARD-12 to its target proteins in a yeast or mammalian two-hybrid system assay. This assay could be automated for high throughput drug screening purposes. In another embodiment of the invention, CARD-12 and a target protein could be configured in the reverse two-hybrid system (Vidal et al. (1996) Proc. Natl. Acad. Sci. USA 93:10321-6 and Vidal et al.  
20 (1996) Proc. Natl. Acad. Sci. USA 93:10315-20) designed specifically for efficient drug screening. In the reverse two-hybrid system, inhibition of a CARD-12 physical interaction with a target protein would result in induction of a reporter gene in contrast to the normal two-hybrid system where inhibition of CARD-12 physical interaction with a target protein would lead to reporter gene repression. The reverse two-hybrid system is  
25 preferred for drug screening because reporter gene induction is more easily assayed than report gene repression.

Alternative embodiments of the invention are proteins found to physically interact with proteins that bind to CARD-12. CARD-12 interactors could be configured into two-hybrid system baits and used in two-hybrid screens to identify additional members of  
30 the CARD-12 pathway. The interactors of CARD-12 interactors identified in this way could be useful targets for therapeutic intervention in CARD-12 related diseases and pathologies and an assay of their enzymatic or binding activity could be useful for the identification of test compounds that modulate CARD-12 activity.

This invention further pertains to novel agents identified by the above-described  
35 screening assays and uses thereof for treatments as described herein.

## **B. Detection Assays**

Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to: (i) map their  
5 respective genes on a chromosome; and, thus, locate gene regions associated with genetic disease; (ii) identify an individual from a minute biological sample (tissue typing); and (iii) aid in forensic identification of a biological sample. These applications are described in the subsections below.

### **1. Chromosome Mapping**

10 Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome. Accordingly, CARD-12 nucleic acid molecules described herein or fragments thereof, can be used to map the location of CARD-12 genes on a chromosome. The mapping of the CARD-12 sequences to chromosomes is an important first step in correlating these sequences with  
15 genes associated with disease.

Briefly, CARD-12 genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the CARD-12 sequences. Computer analysis of CARD-12 sequences can be used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process.  
20 These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the CARD-12 sequences will yield an amplified fragment.

Somatic cell hybrids are prepared by fusing somatic cells from different mammals (e.g., human and mouse cells). As hybrids of human and mouse cells grow and divide,  
25 they gradually lose human chromosomes in random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains the gene encoding the needed enzyme, will be retained. By using various media, panels of hybrid cell lines can be established. Each cell line in a panel contains either a single  
30 human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes. (D'Eustachio et al. (1983) Science 220:919-924). Somatic cell hybrids containing only fragments of human chromosomes can also be produced using human chromosomes with translocations and deletions.

35 PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be

assigned per day using a single thermal cycler. Using the CARD-12 sequences to design oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a CARD-12 sequence to its chromosome include in situ hybridization (described in Fan et al. (1990) Proc. Natl. Acad. Sci. USA 87:6223-27), pre-screening with labeled flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries.

Fluorescence in situ hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. Chromosome spreads can be made using cells whose division has been blocked in metaphase by a chemical like colcemid that disrupts the mitotic spindle. The chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma et al., (Human Chromosomes: A Manual of Basic Techniques (Pergamon Press, New York, 1988)).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between genes and disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, e.g., Egeland et al. (1987) Nature, 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the CARD-12 gene can be determined. If a mutation is observed in some or all of the affected individuals but not in any unaffected

individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

A CARD-12 polypeptide and fragments and sequences thereof and antibodies specific thereto can be used to map the location of the gene encoding the polypeptide on a chromosome. This mapping can be carried out by specifically detecting the presence of the polypeptide in members of a panel of somatic cell hybrids between cells of a first species of animal from which the protein originates and cells from a second species of animal and then determining which somatic cell hybrid(s) expresses the polypeptide and noting the chromosome(s) from the first species of animal that it contains. For examples of this technique, see Pajunen *et al.* (1988) *Cytogenet. Cell Genet.* 47:37-41 and Van Keuren *et al.* (1986) *Hum. Genet.* 74:34-40. Alternatively, the presence of the CARD-12 polypeptide in the somatic cell hybrids can be determined by assaying an activity or property of the polypeptide, for example, enzymatic activity, as described in Bordelon-Riser *et al.* (1979) *Somatic Cell Genetics* 5:597-613 and Owerbach *et al.* (1978) *Proc. Natl. Acad. Sci. USA* 75:5640-5644.

## 2. Tissue Typing

The CARD-12 sequences of the present invention can also be used to identify individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the CARD-12 sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it.

Panels of corresponding DNA sequences from individuals, prepared in this manner, can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences. The sequences of the present invention can be used to obtain such identification sequences from individuals and from tissue. The CARD-12 sequences of the invention uniquely represent portions of the human genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. It is estimated that allelic variation between individual humans occurs with a frequency of about once per each 500 bases. Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared for identification purposes. Because greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals. The noncoding sequences of SEQ ID NO:1 can comfortably provide positive individual identification with a panel of perhaps 10 to 1,000 primers which each yield a noncoding amplified sequence of 100 bases. If predicted coding sequences, such as those in SEQ ID NO:3 are used, a more appropriate number of primers for positive individual identification would be 500-2,000.

If a panel of reagents from CARD-12 sequences described herein is used to generate a unique identification database for an individual, those same reagents can later be used to identify tissue from that individual. Using the unique identification database, positive identification of the individual, living or dead, can be made from extremely small tissue samples.

### **3. Use of Partial Sequences in Forensic Biology**

DNA-based identification techniques can also be used in forensic biology. Forensic biology is a scientific field employing genetic typing of biological evidence found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. To make such an identification, PCR technology can be used to amplify DNA sequences taken from very small biological samples such as tissues, e.g., hair or skin, or body fluids, e.g., blood, saliva, or semen found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide reagents, e.g., PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (i.e. another DNA sequence that is unique to a particular individual). As mentioned above, actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme

generated fragments. Sequences targeted to noncoding regions of SEQ ID NO:1 are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding regions, making it easier to differentiate individuals using this technique. Examples of polynucleotide reagents include the CARD-12 sequences or portions thereof, e.g., fragments derived from the noncoding regions of SEQ ID NO:1 which have a length of at least 20 or 30 bases.

The sequences described herein can further be used to provide polynucleotide reagents, e.g., labeled or labelable probes which can be used in, for example, an in situ hybridization technique, to identify a specific tissue, e.g., brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such CARD-12 probes can be used to identify tissue by species and/or by organ type.

In a similar fashion, these reagents, e.g., CARD-12 primers or probes can be used to screen tissue culture for contamination (i.e., screen for the presence of a mixture of different types of cells in a culture).

### C. Predictive Medicine

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, pharmacogenomics, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining CARD-12 protein and/or nucleic acid expression as well as CARD-12 activity, in the context of a biological sample (e.g., blood, serum, cells, tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant CARD-12 expression or activity. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with CARD-12 protein, nucleic acid expression or activity. For example, mutations in a CARD-12 gene can be assayed in a biological sample. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with CARD-12 protein, nucleic acid expression or activity.

Another aspect of the invention provides methods for determining CARD-12 protein, nucleic acid expression or CARD-12 activity in an individual to thereby select appropriate therapeutic or prophylactic agents for that individual (referred to herein as "pharmacogenomics"). Pharmacogenomics allows for the selection of agents (e.g., drugs) for therapeutic or prophylactic treatment of an individual based on the genotype of

the individual (e.g., the genotype of the individual examined to determine the ability of the individual to respond to a particular agent.)

Yet another aspect of the invention pertains to monitoring the influence of agents (e.g., drugs or other compounds) on the expression or activity of CARD-12 in clinical trials.

These and other agents are described in further detail in the following sections.

#### 1. Diagnostic Assays

An exemplary method for detecting the presence or absence of CARD-12 in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting CARD-12 protein or nucleic acid (e.g., mRNA, genomic DNA) that encodes CARD-12 protein such that the presence of CARD-12 is detected in the biological sample. An agent for detecting CARD-12 mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to CARD-12 mRNA or genomic DNA. The nucleic acid probe can be, for example, a full-length CARD-12 nucleic acid, such as the nucleic acid of SEQ ID NO:1 or 3, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to mRNA or genomic DNA. Other suitable probes for use in the diagnostic assays of the invention are described herein.

An agent for detecting CARD-12 protein can be an antibody capable of binding to CARD-12 protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or F(ab')<sub>2</sub>) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect CARD-12 mRNA, protein, or genomic DNA in a biological sample in vitro as well as in vivo. For example, in vitro techniques for detection of CARD-12 mRNA include Northern hybridizations and in situ hybridizations. In vitro techniques for detection of CARD-12 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and

immunofluorescence. In vitro techniques for detection of CARD-12 genomic DNA include Southern hybridizations. Furthermore, in vivo techniques for detection of CARD-12 protein include introducing into a subject a labeled anti-CARD-12 antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one embodiment, the biological sample contains protein molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject. A biological sample is a peripheral blood leukocyte sample isolated by conventional means from a subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting CARD-12 protein, mRNA, or genomic DNA, such that the presence of CARD-12 protein, mRNA or genomic DNA is detected in the biological sample, and comparing the presence of CARD-12 protein, mRNA or genomic DNA in the control sample with the presence of CARD-12 protein, mRNA or genomic DNA in the test sample.

The invention also encompasses kits for detecting the presence of CARD-12 in a biological sample (a test sample). Such kits can be used to determine if a subject is suffering from or is at increased risk of developing a disorder associated with aberrant expression of CARD-12 (e.g., an immunological disorder). For example, the kit can comprise a labeled compound or agent capable of detecting CARD-12 protein or mRNA in a biological sample and means for determining the amount of CARD-12 in the sample (e.g., an anti-CARD-12 antibody or an oligonucleotide probe which binds to DNA-encoding CARD-12, e.g., SEQ ID NO:1 or SEQ ID NO:3). Kits may also include instruction for observing that the tested subject is suffering from or is at risk of developing a disorder associated with aberrant expression of CARD-12 if the amount of CARD-12 protein or mRNA is above or below a normal level.

For antibody-based kits, the kit may comprise, for example: (1) a first antibody (e.g., attached to a solid support) which binds to CARD-12 protein; and, optionally, (2) a second, different antibody which binds to CARD-12 protein or the first antibody and is conjugated to a detectable agent. For oligonucleotide-based kits, the kit may comprise, for example: (1) a oligonucleotide, e.g., a detectably labelled oligonucleotide, which hybridizes to a CARD-12 nucleic acid sequence or (2) a pair of primers useful for amplifying a CARD-12 nucleic acid molecule.

The kit may also comprise, e.g., a buffering agent, a preservative, or a protein stabilizing agent. The kit may also comprise components necessary for detecting the



detectable agent (e.g., an enzyme or a substrate). The kit may also contain a control sample or a series of control samples which can be assayed and compared to the test sample contained. Each component of the kit is usually enclosed within an individual container and all of the various containers are within a single package along with instructions for observing whether the tested subject is suffering from or is at risk of developing a disorder associated with aberrant expression of CARD-12.

## 2. Prognostic Assays

The methods described herein can furthermore be utilized as diagnostic or prognostic assays to identify subjects having or at risk of developing a disease or disorder associated with aberrant CARD-12 expression or activity. For example, the assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a disorder associated with CARD-12 protein, nucleic acid expression or activity. Alternatively, the prognostic assays can be utilized to identify a subject having or at risk for developing such a disease or disorder. Thus, the present invention provides a method in which a test sample is obtained from a subject and CARD-12 protein or nucleic acid (e.g., mRNA, genomic DNA) is detected, wherein the presence of CARD-12 protein or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant CARD-12 expression or activity. As used herein, a "test sample" refers to a biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (e.g., serum), cell sample, or tissue. Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant CARD-12 expression or activity. For example, such methods can be used to determine whether a subject can be effectively treated with a specific agent or class of agents (e.g., agents of a type which decrease CARD-12 activity). Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder associated with aberrant CARD-12 expression or activity in which a test sample is obtained and CARD-12 protein or nucleic acid is detected (e.g., wherein the presence of CARD-12 protein or nucleic acid is diagnostic for a subject that can be administered the agent to treat a disorder associated with aberrant CARD-12 expression or activity).

The methods of the invention can also be used to detect genetic lesions or mutations in a CARD-12 gene, thereby determining if a subject with the lesioned gene is at risk for a disorder characterized by aberrant cell proliferation and/or differentiation. In preferred embodiments, the methods include detecting, in a sample of cells from the

subject, the presence or absence of a genetic lesion characterized by at least one of an alteration affecting the integrity of a gene encoding a CARD-12-protein, or the mis-expression of the CARD-12 gene. For example, such genetic lesions can be detected by CARD-5ertaining the existence of at least one of 1) a deletion of one or more  
5 nucleotides from a CARD-12 gene; 2) an addition of one or more nucleotides to a CARD-12 gene; 3) a substitution of one or more nucleotides of a CARD-12 gene; 4) a chromosomal rearrangement of a CARD-12 gene; 5) an alteration in the level of a messenger RNA transcript of a CARD-12 gene; 6) aberrant modification of a CARD-12 gene, such as of the methylation pattern of the genomic DNA; 7) the presence of a  
10 non-wild type splicing pattern of a messenger RNA transcript of a CARD-12 gene (e.g., caused by a mutation in a splice donor or splice acceptor site); 8) a non-wild type level of a CARD-12-protein; 9) allelic loss of a CARD-12 gene; and 10) inappropriate post-translational modification of a CARD-12-protein. As described herein, there are a large number of assay techniques known in the art which can be used for detecting  
15 lesions in a CARD-12 gene. A biological sample is a peripheral blood leukocyte sample isolated by conventional means from a subject.

In certain embodiments, detection of the lesion involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain  
20 reaction (LCR) (see, e.g., Landegran et al. (1988) Science 241:1077-1080; and Nakazawa et al. (1994) Proc. Natl. Acad. Sci. USA 91:360-364), the latter of which can be particularly useful for detecting point mutations in the CARD-12 gene (see, e.g., Abravaya et al. (1995) Nucleic Acids Res. 23:675-682). This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (e.g., genomic,  
25 mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a CARD-12 gene under conditions such that hybridization and amplification of the CARD-12-gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that  
30 PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self sustained sequence replication (Guatelli et al. (1990) Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh, et al. (1989) Proc. Natl. Acad. Sci. USA 86:1173-1177),  
35 Q-Beta Replicase (Lizardi et al. (1988) Bio/Technology 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using

techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

5 In an alternative embodiment, mutations in a CARD-12 gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence  
10 specific ribozymes (see, e.g., U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in CARD-12 can be identified by hybridizing a sample and control nucleic acids, e.g., DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin et al. (1996) Human  
15 Mutation 7:244-255; Kozal et al. (1996) Nature Medicine 2:753-759). For example, genetic mutations in CARD-12 can be identified in two-dimensional arrays containing light-generated DNA probes as described in Cronin et al. supra. Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear  
20 arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets; one complementary to the wild-type gene and the other  
25 complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the CARD-12 gene and detect mutations by comparing the sequence of the sample CARD-12 with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques  
30 developed by Maxam and Gilbert ((1977) Proc. Natl. Acad. Sci. USA 74:560) or Sanger ((1977) Proc. Natl. Acad. Sci. USA 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) Bio/Techniques 19:448), including sequencing by mass spectrometry (see, e.g., PCT Publication No. WO 94/16101; Cohen et al. (1996) Adv.  
35 Chromatogr. 36:127-162; and Griffin et al. (1993) Appl. Biochem. Biotechnol. 38:147-159).

Other methods for detecting mutations in the CARD-12 gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers et al. (1985) Science 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes of formed by  
5 hybridizing (labeled) RNA or DNA containing the wild-type CARD-12 sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA  
10 hybrids treated with S1 nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation.  
15 See, e.g., Cotton et al (1988) Proc. Natl Acad Sci USA 85:4397; Saleeba et al (1992) Methods Enzymol. 217:286-295. In an embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations  
20 in CARD-12 cDNAs obtained from samples of cells. For example, the mutY enzyme of E. coli cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu et al. (1994) Carcinogenesis 15:1657-1662). According to an exemplary embodiment, a probe based on a CARD-12 sequence, e.g., a  
25 wild-type CARD-12 sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, e.g., U.S. Patent No. 5,459,039.

In other embodiments, alterations in electrophoretic mobility will be used to  
30 identify mutations in CARD-12 genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al. (1989) Proc Natl. Acad. Sci USA: 86:2766, see also Cotton (1993) Mutat. Res. 285:125-144; and Hayashi (1992) Genet Anal Tech Appl 9:73-79). Single-stranded DNA fragments of sample and control  
35 CARD-12 nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting

alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In an embodiment, the subject  
5 method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al. (1991) Trends Genet 7:5).

In yet another embodiment, the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing  
10 gradient gel electrophoresis (DGGE) (Myers et al. (1985) Nature 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control  
15 and sample DNA (Rosenbaum and Reissner (1987) Biophys Chem 265:12753).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions  
20 which permit hybridization only if a perfect match is found (Saiki et al. (1986) Nature 324:163); Saiki et al. (1989) Proc. Natl Acad. Sci USA 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective  
25 PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs et al. (1989) Nucleic Acids Res. 17:2437-2448) or at the extreme 3'  
30 end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) Tibtech 11:238). In addition, it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini et al. (1992) Mol. Cell Probes 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification  
35 (Barany (1991) Proc. Natl. Acad. Sci USA 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect

the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, e.g., in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a CARD-12 gene.

Furthermore, any cell type or tissue, preferably peripheral blood leukocytes, in which CARD-12 is expressed may be utilized in the prognostic assays described herein.

### 3. Pharmacogenomics

Agents, or modulators which have a stimulatory or inhibitory effect on CARD-12 activity (e.g., CARD-12 gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) disorders (e.g., an immunological disorder) associated with aberrant CARD-12 activity. In conjunction with such treatment, the pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) of the individual may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, the pharmacogenomics of the individual permits the selection of effective agents (e.g., drugs) for prophylactic or therapeutic treatments based on a consideration of the individual's genotype. Such pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens. Accordingly, the activity of CARD-12 protein, expression of CARD-12 nucleic acid, or mutation content of CARD-12 genes in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual.

Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, e.g., Linder (1997) Clin. Chem. 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare defects or as polymorphisms. For example, glucose-6-phosphate dehydrogenase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is

haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

As an illustrative embodiment, the activity of drug-metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug-metabolizing enzymes (e.g., N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM exhibit no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so-called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Thus, the activity of CARD-12 protein, expression of CARD-12 nucleic acid, or mutation content of CARD-12 genes in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual. In addition, pharmacogenetic studies can be used to apply genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the identification of an individual's drug responsiveness phenotype. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a CARD-12 modulator, such as a modulator identified by one of the exemplary screening assays described herein.

#### **4. Monitoring of Effects During Clinical Trials**

Monitoring the influence of agents (e.g., drugs, compounds) on the expression or activity of CARD-12 (e.g., the ability to modulate aberrant cell proliferation and/or differentiation) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase CARD-12 gene expression, protein levels, or upregulate CARD-12 activity, can be monitored in clinical trials of subjects exhibiting decreased CARD-12 gene expression, protein levels, or downregulated CARD-12 activity. Alternatively, the

effectiveness of an agent determined by a screening assay to decrease CARD-12 gene expression, protein levels, or downregulated CARD-12 activity, can be monitored in clinical trials of subjects exhibiting increased CARD-12 gene expression, protein levels, or upregulated CARD-12 activity. In such clinical trials, the expression or activity of  
5 CARD-12 and, preferably, other genes that have been implicated in, for example, a cellular proliferation disorder can be used as a "read out" or markers of the immune responsiveness of a particular cell.

For example, and not by way of limitation, genes, including CARD-12, that are modulated in cells by treatment with an agent (e.g., compound, drug or small molecule)  
10 which modulates CARD-12 activity (e.g., identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on cellular proliferation disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of CARD-12 and other genes implicated in the disorder. The levels of gene expression (i.e., a gene expression pattern) can be quantified  
15 by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of CARD-12 or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at  
20 various points during, treatment of the individual with the agent.

In an embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining  
25 a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a CARD-12 protein, mRNA, or genomic DNA in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the CARD-12 protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of  
30 expression or activity of the CARD-12 protein, mRNA, or genomic DNA in the pre-administration sample with the CARD-12 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of CARD-12 to higher levels than  
35 detected, i.e., to increase the effectiveness of the agent. Alternatively, decreased



administration of the agent may be desirable to decrease expression or activity of CARD-12 to lower levels than detected, i.e., to decrease the effectiveness of the agent.

### 5. Transcriptional Profiling

The CARD-12 nucleic acid molecules described herein, including small oligonucleotides, can be used in transcriptionally profiling. For example, these nucleic acids can be used to examine the expression of CARD-12 in normal tissue or cells and in tissue or cells subject to a disease state, e.g., tissue or cells derived from a patient having a disease of interest or cultured cells which model or reflect a disease state of interest, e.g., cells of a cultured tumor cell line. By measuring expression of CARD-12, together or individually, a profile of expression in normal and disease states can be developed. This profile can be used diagnostically and to examine the effectiveness of a therapeutic regime.

### C. Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant CARD-12 expression or activity, examples of which are provided herein.

#### 1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant CARD-12 expression or activity, by administering to the subject an agent which modulates CARD-12 expression or at least one CARD-12 activity. Subjects at risk for a disease which is caused or contributed to by aberrant CARD-12 expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the CARD-12 aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of CARD-12 aberrancy, for example, a CARD-12 agonist or CARD-12 antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

#### 2. Therapeutic Methods

Another aspect of the invention pertains to methods of modulating CARD-12 expression or activity for therapeutic purposes. The modulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of CARD-12 protein activity associated with the cell. An agent that modulates CARD-12 protein activity can be an agent as described herein, such as a nucleic acid or a protein, a naturally-occurring cognate ligand of a CARD-12 protein, a peptide, a CARD-12

peptidomimetic, or other small molecule. In one embodiment, the agent stimulates one or more of the biological activities of CARD-12 protein. Examples of such stimulatory agents include active CARD-12 protein and a nucleic acid molecule encoding CARD-12 that has been introduced into the cell. In another embodiment, the agent inhibits one or more of the biological activities of CARD-12 protein. Examples of such inhibitory agents include antisense CARD-12 nucleic acid molecules and anti-CARD-12 antibodies. These modulatory methods can be performed in vitro (e.g., by culturing the cell with the agent) or, alternatively, in vivo (e.g., by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant expression or activity of a CARD-12 protein or nucleic acid molecule or a disorder related to CARD-12 expression or activity. In one embodiment, the method involves administering an agent (e.g., an agent identified by a screening assay described herein), or combination of agents that modulates (e.g., upregulates or downregulates) CARD-12 expression or activity. In another embodiment, the method involves administering a CARD-12 protein or nucleic acid molecule as therapy to compensate for reduced or aberrant CARD-12 expression or activity.

Stimulation of CARD-12 activity is desirable in situations in which CARD-12 is abnormally downregulated and/or in which increased CARD-12 activity is likely to have a beneficial effect. Conversely, inhibition of CARD-12 activity is desirable in situations in which CARD-12 is abnormally upregulated, e.g., in myocardial infarction, and/or in which decreased CARD-12 activity is likely to have a beneficial effect.

### Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. An isolated nucleic acid molecule comprising at least 50 contiguous nucleotides of SEQ ID NO:1.
- 5 2. The isolated nucleic acid molecule of claim 1 wherein the nucleic acid molecule comprises at least 150 contiguous nucleotide of SEQ ID NO:1.
3. The isolated nucleic acid molecule of claim 1 wherein the nucleic acid  
10 molecule comprises at least 500 contiguous nucleotide of SEQ ID NO:1.
4. An isolated nucleic acid molecule comprising at least 50 contiguous nucleotides of SEQ ID NO:3.
- 15 5. The isolated nucleic acid molecule of claim 4 wherein the nucleic acid molecule comprises at least 150 contiguous nucleotide of SEQ ID NO:3.
6. The isolated nucleic acid molecule of claim 4 wherein the nucleic acid molecule comprises at least 500 contiguous nucleotide of SEQ ID NO:3.
- 20 7. The isolated nucleic acid molecule of claim 1 wherein the nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO:1.
8. The isolated nucleic acid molecule of claim 1 wherein the nucleic acid  
25 molecule comprises the nucleotide sequence of SEQ ID NO:3.
9. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising at least 25 contiguous amino acids of SEQ ID NO:2.
- 30 10. The isolated nucleic acid molecule of claim 9 wherein the polypeptide comprises at least 150 contiguous amino acids of SEQ ID NO:2.

11. The isolated nucleic acid molecule of claim 9 wherein the polypeptide comprises at least one domain selected from the group consisting of a CARD domain, a NACHT domain, a leucine rich repeat domain, and an NBS domain.
- 5 12. The isolated nucleic acid molecule of claim 9 wherein the polypeptide comprises at least two domains selected from the group consisting of a CARD domain, a NACHT motif domain, a leucine rich repeat domain, and an NBS domain.
- 10 13. The isolated nucleic acid molecule of claim 9 wherein the polypeptide comprises at least two NACHT motif domains.
14. The isolated nucleic acid molecule of claim 9 wherein the polypeptide comprises at least two leucine rich repeat domains.
- 15 15. The isolated nucleic acid molecule of claim 9 wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:2.
16. An isolated nucleic acid molecule that hybridizes to a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:3 under conditions of incubation at 45°C in 6.0X SSC followed by washing in 0.2X SSC/0.1% SDS at 65°C.
- 20 17. The nucleic acid molecule of claim 1 further comprising vector nucleic acid sequences.
18. The nucleic acid molecule of claim 17 wherein the vector nucleic acid sequences comprise expression control sequences.
- 25 19. A host cell that contains the nucleic acid molecule of claim 1.
20. The host cell of claim 19 wherein the host cell is a mammalian cell.
21. An isolated polypeptide comprising at least 15 contiguous amino acids of SEQ ID NO:2.

22. The isolated polypeptide of claim 21 wherein the polypeptide comprises at least 75 contiguous amino acids of SEQ ID NO:2.

23. The isolated polypeptide of claim 21 comprising the amino acid sequence of SEQ ID NO:2

5 24. An isolated polypeptide comprising the amino acid sequence of SEQ ID NO:2 wherein up to 10 amino acids are replaced by conservative substitutions.

25. A fusion protein comprising at least 25 contiguous amino acids of SEQ ID NO:2

10 26. An antibody which selectively binds to a polypeptide consisting of the amino acid sequence of SEQ IN NO:2.

27. A method for detecting the presence of a polypeptide of claim 21 in a sample, comprising:

- 15 a) contacting the sample with a compound which selectively binds to a polypeptide of claim 2; and  
b) determining whether the compound binds to the polypeptide in the sample.

28. The method of claim 27, wherein the compound that binds to the polypeptide is an antibody.

29. A kit comprising a compound that selectively binds to a polypeptide of claim 22 and instructions for use.

20 30. A method for detecting the presence of a nucleic acid molecule of claim 1 in a sample, comprising the steps of:

- a) contacting the sample with a nucleic acid probe or primer which selectively hybridizes to the nucleic acid molecule of claim 1; and  
25 b) determining whether the nucleic acid probe or primer binds to a nucleic acid molecule in the sample.

31. The method of claim 30, wherein the sample comprises mRNA molecules and is contacted with a nucleic acid probe.

32. A kit comprising a compound that selectively hybridizes to a nucleic acid molecule of claim 1 and instructions for use.

33. A method for identifying a compound which binds to a polypeptide of comprising the amino acid sequence of SEQ ID NO:2 the method comprising the steps of:

- 5 a) contacting a polypeptide, or a cell expressing a polypeptide comprising the amino acid sequence of SEQ ID NO:2 with a test compound; and  
b) determining whether the polypeptide binds to the test compound.

34. The method of claim 33, wherein the binding of the test compound to the polypeptide is detected by a method selected from the group consisting of:

- 10 a) detection of binding by direct detecting of test compound/polypeptide binding;  
b) detection of binding using a competition binding assay;  
c) detection of binding using an assay for CARD-12-mediated signal transduction; and  
d) detection of binding of a CARD-12 to a CARD domain.

15 35. A method for identifying a compound that modulates the activity of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, comprising:

- a) contacting a polypeptide of claim 21 with a test compound; and  
b) determining the effect of the test compound on the activity of the polypeptide to thereby identify a compound which modulates the activity of the  
20 polypeptide.

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cgctctagcc cggtgggaag ctttcatcca gaaca atg aat ttc ata aag gac	53
Met Asn Phe Ile Lys Asp	
1 5	
aat agc cga gcc ctt att caa aga atg gga atg act gtt ata aag caa	101
Asn Ser Arg Ala Leu Ile Gln Arg Met Gly Met Thr Val Ile Lys Gln	
10 15 20	
atc aca gat gac cta ttt gta tgg aat gtt ctg aat cgc gaa gaa gta	149
Ile Thr Asp Asp Leu Phe Val Trp Asn Val Leu Asn Arg Glu Glu Val	
25 30 35	
aac atc att tgc tgc gag aag gtg gag cag gat gct gct aga ggg atc	197
Asn Ile Ile Cys Cys Glu Lys Val Glu Gln Asp Ala Ala Arg Gly Ile	
40 45 50	
att cac atg att ttg aaa aag ggt tca gag tcc tgt aac ctc ttt ctt	245
Ile His Met Ile Leu Lys Lys Gly Ser Glu Ser Cys Asn Leu Phe Leu	
55 60 65 70	
aaa tcc ctt aag gag tgg aac tat cct cta ttt cag gac ttg aat gga	293
Lys Ser Leu Lys Glu Trp Asn Tyr Pro Leu Phe Gln Asp Leu Asn Gly	
75 80 85	
caa agt ctt ttt cat cag aca tca gaa gga gac ttg gac gat ttg gct	341
Gln Ser Leu Phe His Gln Thr Ser Glu Gly Asp Leu Asp Asp Leu Ala	
90 95 100	
cag gat tta aag gac ttg tac cat acc cca tct ttt ctg aac ttt tat	389
Gln Asp Leu Lys Asp Leu Tyr His Thr Pro Ser Phe Leu Asn Phe Tyr	
105 110 115	
ccc ctt ggt gaa gat att gac att att ttt aac ttg aaa agc acc ttc	437
Pro Leu Gly Glu Asp Ile Asp Ile Ile Phe Asn Leu Lys Ser Thr Phe	
120 125 130	
aca gaa cct gtc ctg tgg agg aag gac caa cac cat cac cgc gtg gag	485
Thr Glu Pro Val Leu Trp Arg Lys Asp Gln His His His Arg Val Glu	
135 140 145 150	
cag ctg acc ctg aat ggc ctc ctg cag gct ctt cag agc ccc tgc atc	533
Gln Leu Thr Leu Asn Gly Leu Leu Gln Ala Leu Gln Ser Pro Cys Ile	
155 160 165	
att gaa ggg gaa tct ggc aaa ggc aag tcc act ctg ctg cag cgc att	581
Ile Glu Gly Glu Ser Gly Lys Gly Lys Ser Thr Leu Leu Gln Arg Ile	
170 175 180	
gcc atg ctc tgg ggc tcc gga aag tgc aag gct ctg acc aag ttc aaa	629
Ala Met Leu Trp Gly Ser Gly Lys Cys Lys Ala Leu Thr Lys Phe Lys	
185 190 195	
ttc gtc ttc ttc ctc cgt ctc agc agg gcc cag ggt gga ctt ttt gaa	677
Phe Val Phe Phe Leu Arg Leu Ser Arg Ala Gln Gly Gly Leu Phe Glu	
200 205 210	

FIG. 1A

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acc	ctc	tgt	gat	caa	ctc	ctg	gat	ata	cct	ggc	aca	atc	agg	aag	cag	725
Thr	Leu	Cys	Asp	Gln	Leu	Leu	Asp	Ile	Pro	Gly	Thr	Ile	Arg	Lys	Gln	
215					220					225					230	
aca	ttc	atg	gcc	atg	ctg	ctg	aag	ctg	cgg	cag	agg	gtt	ctt	ttc	ctt	773
Thr	Phe	Met	Ala	Met	Leu	Leu	Lys	Leu	Arg	Gln	Arg	Val	Leu	Phe	Leu	
				235					240					245		
ctt	gat	ggc	tac	aat	gaa	ttc	aag	ccc	cag	aac	tgc	cca	gaa	atc	gaa	821
Leu	Asp	Gly	Tyr	Asn	Glu	Phe	Lys	Pro	Gln	Asn	Cys	Pro	Glu	Ile	Glu	
			250					255					260			
gcc	ctg	ata	aag	gaa	aac	cac	cgc	ttc	aag	aac	atg	gtc	atc	gtc	acc	869
Ala	Leu	Ile	Lys	Glu	Asn	His	Arg	Phe	Lys	Asn	Met	Val	Ile	Val	Thr	
		265					270					275				
act	acc	act	gag	tgc	ctg	agg	cac	ata	cgg	cag	ttt	ggt	gcc	ctg	act	917
Thr	Thr	Thr	Glu	Cys	Leu	Arg	His	Ile	Arg	Gln	Phe	Gly	Ala	Leu	Thr	
	280					285					290					
gct	gag	gtg	ggg	gat	atg	aca	gaa	gac	agc	gcc	cag	gct	ctc	atc	cga	965
Ala	Glu	Val	Gly	Asp	Met	Thr	Glu	Asp	Ser	Ala	Gln	Ala	Leu	Ile	Arg	
295					300					305					310	
gaa	gtg	ctg	atc	aag	gag	ctt	gct	gaa	ggc	ttg	ttg	ctc	caa	att	cag	1013
Glu	Val	Leu	Ile	Lys	Glu	Leu	Ala	Glu	Gly	Leu	Leu	Leu	Gln	Ile	Gln	
				315					320					325		
aaa	tcc	agg	tgc	ttg	agg	aat	ctc	atg	aag	acc	cct	ctc	ttt	gtg	gtc	1061
Lys	Ser	Arg	Cys	Leu	Arg	Asn	Leu	Met	Lys	Thr	Pro	Leu	Phe	Val	Val	
			330					335					340			
atc	act	tgt	gca	atc	cag	atg	ggg	gaa	agt	gag	ttc	cac	tct	cac	aca	1109
Ile	Thr	Cys	Ala	Ile	Gln	Met	Gly	Glu	Ser	Glu	Phe	His	Ser	His	Thr	
		345					350					355				
caa	aca	acg	ctg	ttc	cat	acc	ttc	tat	gat	ctg	ttg	ata	cag	aaa	aac	1157
Gln	Thr	Thr	Leu	Phe	His	Thr	Phe	Tyr	Asp	Leu	Leu	Ile	Gln	Lys	Asn	
	360					365					370					
aaa	cac	aaa	cat	aaa	ggg	gtg	gct	gca	agt	gac	ttc	att	cgg	agc	ctg	1205
Lys	His	Lys	His	Lys	Gly	Val	Ala	Ala	Ser	Asp	Phe	Ile	Arg	Ser	Leu	
375					380					385					390	
gac	cac	tgt	gga	gac	cta	gct	ctg	gag	ggg	gtg	ttc	tcc	cac	aag	ttt	1253
Asp	His	Cys	Gly	Asp	Leu	Ala	Leu	Glu	Gly	Val	Phe	Ser	His	Lys	Phe	
				395					400					405		
gat	ttc	gaa	ctg	cag	gat	gtg	tcc	agc	gtg	aat	gag	gat	gtc	ctg	ctg	1301
Asp	Phe	Glu	Leu	Gln	Asp	Val	Ser	Ser	Val	Asn	Glu	Asp	Val	Leu	Leu	
			410					415								

**FIG. 1B**



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tat aaa ttc ttt cac aag tca ttc cag gag tac aca gca gga cga aga Tyr Lys Phe Phe His Lys Ser Phe Gln Glu Tyr Thr Ala Gly Arg Arg 440 445 450	1397
ctc agc agt tta ttg acg tct cat gag cca gag gag gtg acc aag ggg Leu Ser Ser Leu Leu Thr Ser His Glu Pro Glu Glu Val Thr Lys Gly 455 460 465 470	1445
aat ggt tac ttg cag aaa atg gtt tcc att tcg gac att aca tcc act Asn Gly Tyr Leu Gln Lys Met Val Ser Ile Ser Asp Ile Thr Ser Thr 475 480 485	1493
tat agc agc ctg ctc cgg tac acc tgt ggg tca tct gtg gaa gcc acc Tyr Ser Ser Leu Leu Arg Tyr Thr Cys Gly Ser Ser Val Glu Ala Thr 490 495 500	1541
agg gct gtt atg aag cac ctc gca gca gtg tat caa cac ggc tgc ctt Arg Ala Val Met Lys His Leu Ala Ala Val Tyr Gln His Gly Cys Leu 505 510 515	1589
ctc gga ctt tcc atc gcc aag agg cct ctc tgg aga cag gaa tct ttg Leu Gly Leu Ser Ile Ala Lys Arg Pro Leu Trp Arg Gln Glu Ser Leu 520 525 530	1637
caa agt gtg aaa aac acc act gag caa gaa att ctg aaa gcc ata aac Gln Ser Val Lys Asn Thr Thr Glu Gln Glu Ile Leu Lys Ala Ile Asn 535 540 545 550	1685
atc aat tcc ttt gta gag tgt ggc atc cat tta tat caa gag agt aca Ile Asn Ser Phe Val Glu Cys Gly Ile His Leu Tyr Gln Glu Ser Thr 555 560 565	1733
tcc aaa tca gcc ctg agc caa gaa ttt gaa gct ttc ttt caa ggt aaa Ser Lys Ser Ala Leu Ser Gln Glu Phe Glu Ala Phe Phe Gln Gly Lys 570 575 580	1781
agc tta tat atc aac tca ggg aac atc ccc gat tac tta ttt gac ttc Ser Leu Tyr Ile Asn Ser Gly Asn Ile Pro Asp Tyr Leu Phe Asp Phe 585 590 595	1829
ttt gaa cat ttg ccc aat tgt gca agt gct ctg gac ttc att aaa ctg Phe Glu His Leu Pro Asn Cys Ala Ser Ala Leu Asp Phe Ile Lys Leu 600 605 610	1877
gac ttt tat ggg gga gct atg gct tca tgg gaa aag gct gca gaa gac Asp Phe Tyr Gly Gly Ala Met Ala Ser Trp Glu Lys Ala Ala Glu Asp 615 620 625 630	1925
aca ggt gga atc cac atg gaa gag gcc cca gaa acc tac att ccc agc Thr Gly Gly Ile His Met Glu Glu Ala Pro Glu Thr Tyr Ile Pro Ser 635 640 645	1973
agg gct gta tct ttg ttc ttc aac tgg aag cag gaa ttc agg act ctg Arg Ala Val Ser Leu Phe Phe Asn Trp Lys Gln Glu Phe Arg Thr Leu 650 655 660	2021

FIG. 1C

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gag gtc aca ctc cgg gat ttc agc aag ttg aat aag caa gat atc aca Glu Val Thr Leu Arg Asp Phe Ser Lys Leu Asn Lys Gln Asp Ile Thr 665 670 675	2069
tat ctg ggg aaa ata ttc agc tct gcc aca agc ctc agg ctg caa ata Tyr Leu Gly Lys Ile Phe Ser Ser Ala Thr Ser Leu Arg Leu Gln Ile 680 685 690	2117
aag aga tgt gct ggt gtg gct gga agc ctc agt ttg gtc ctc agc acc Lys Arg Cys Ala Gly Val Ala Gly Ser Leu Ser Leu Val Leu Ser Thr 695 700 705 710	2165
tgt aag aac att tat tct ctc atg gtg gaa gcc agt ccc ctc acc ata Cys Lys Asn Ile Tyr Ser Leu Met Val Glu Ala Ser Pro Leu Thr Ile 715 720 725	2213
gaa gat gag agg cac atc aca tct gta aca aac ctg aaa acc ttg agt Glu Asp Glu Arg His Ile Thr Ser Val Thr Asn Leu Lys Thr Leu Ser 730 735 740	2261
att cat gac cta cag aat caa cgg ctg ccg ggt ggt ctg act gac agc Ile His Asp Leu Gln Asn Gln Arg Leu Pro Gly Gly Leu Thr Asp Ser 745 750 755	2309
ttg ggt aac ttg aag aac ctt aca aag ctc ata atg gat aac ata aag Leu Gly Asn Leu Lys Asn Leu Thr Lys Leu Ile Met Asp Asn Ile Lys 760 765 770	2357
atg aat gaa gaa gat gct ata aaa cta gct gaa ggc ctg aaa aac ctg Met Asn Glu Glu Asp Ala Ile Lys Leu Ala Glu Gly Leu Lys Asn Leu 775 780 785 790	2405
aag aag atg tgt tta ttt cat ttg acc cac ttg tct gac att gga gag Lys Lys Met Cys Leu Phe His Leu Thr His Leu Ser Asp Ile Gly Glu 795 800 805	2453
gga atg gat tac ata gtc aag tct ctg tca agt gaa ccc tgt gac ctt Gly Met Asp Tyr Ile Val Lys Ser Leu Ser Ser Glu Pro Cys Asp Leu 810 815 820	2501
gaa gaa att caa tta gtc tcc tgc tgc ttg tct gca aat gca gtg aaa Glu Glu Ile Gln Leu Val Ser Cys Cys Leu Ser Ala Asn Ala Val Lys 825 830 835	2549
atc cta gct cag aat ctt cac aat ttg gtc aaa ctg agc att ctt gat Ile Leu Ala Gln Asn Leu His Asn Leu Val Lys Leu Ser Ile Leu Asp 840 845 850	2597
tta tca gaa aat tac ctg gaa aaa gat gga aat gaa gct ctt cat gaa Leu Ser Glu Asn Tyr Leu Glu Lys Asp Gly Asn Glu Ala Leu His Glu 855 860 865 870	2645
ctg atc gac agg atg aac gtg cta gaa cag ctc acc gca ctg atg ctg Leu Ile Asp Arg Met Asn Val Leu Glu Gln Leu Thr Ala Leu Met Leu 875 880 885	2693

FIG. 1D

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ccc tgg ggc tgt gac gtg caa ggc agc ctg agc agc ctg ttg aaa cat	2741
Pro Trp Gly Cys Asp Val Gln Gly Ser Leu Ser Ser Leu Leu Lys His	
890 895 900	
ttg gag gag gtc cca caa ctc gtc aag ctt ggg ttg aaa aac tgg aga	2789
Leu Glu Glu Val Pro Gln Leu Val Lys Leu Gly Leu Lys Asn Trp Arg	
905 910 915	
ctc aca gat aca gag att aga att tta ggt gca ttt ttt gga aag aac	2837
Leu Thr Asp Thr Glu Ile Arg Ile Leu Gly Ala Phe Phe Gly Lys Asn	
920 925 930	
cct ctg aaa aac ttc cag cag ttg aat ttg gcg gga aat cgt gtg agc	2885
Pro Leu Lys Asn Phe Gln Gln Leu Asn Leu Ala Gly Asn Arg Val Ser	
935 940 945 950	
agt gat gga tgg ctt gcc ttc atg ggt gta ttt gag aat ctt aag caa	2933
Ser Asp Gly Trp Leu Ala Phe Met Gly Val Phe Glu Asn Leu Lys Gln	
955 960 965	
tta gtg ttt ttt gac ttt agt act aaa gaa ttt cta cct gat cca gca	2981
Leu Val Phe Phe Asp Phe Ser Thr Lys Glu Phe Leu Pro Asp Pro Ala	
970 975 980	
tta gtc aga aaa ctt agc caa gtg tta tcc aag tta act ttt ctg caa	3029
Leu Val Arg Lys Leu Ser Gln Val Leu Ser Lys Leu Thr Phe Leu Gln	
985 990 995	
gaa gct agg ctt gtt ggg tgg caa ttt gat gat gat gat ctc agt gtt	3077
Glu Ala Arg Leu Val Gly Trp Gln Phe Asp Asp Asp Asp Leu Ser Val	
1000 1005 1010	
att aca ggt gct ttt aaa cta gta act gct taa ataaagtgtgta ctcgaagcca	3130
Ile Thr Gly Ala Phe Lys Leu Val Thr Ala *	
1015 1020	
gta	3133

FIG. 1E

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1 ATGCTGAACGCTGGTCCCCCTGGGCTCCCTTATTTCTTTCTCTATACTTTGTCTCTGTGTCTTTTTCTTTT  
TACGACTTGCGACCGGACCCGAGGGAATAAAGAAAGAGATATGAAACAGAGACACAGAAAAAGAAAA  
1▶ M L N A G P L G S L I S F S I L C L C V F F F  
24 CCAAGTCTCTCGTTCCACCTAACGAGAAACACCCACAGAACAAGAAGGTATCTGGTCTACAAGAACTCGA  
GGTTCAGAGAGCAAGGTGGATTGCTCTTTGTGGGTGTCTGTCTTCCATAGACCAGATGTTCTTGAGCT  
24▶ S K S L V P P N E K H P Q N K K V S G L Q E L E  
141 GGCCTCACTGAAACGGAAAGCAAATACAAAGAACTTTATTTTAAAAACATGTCTTGGTCTCCCAAGAAG  
CCGGAGTGACTTTGCCTTTCTGTTTATGTTTCTTTGAAATAAAATTTTGTACAGAACCAGAGGGTTCTTC  
47▶ A S L K R K A N T K K L Y F K N M S W S P K K  
211 AGGGCAATTGGATTGCTCAGCCAGAGACCCCTTGAGGCAGACACACAAGCGGCTGGACGTCGAGAGGAAC  
TCCCGTTAACCTAACGAGTCGGTCTCTGGGAACGTCCGTCTGTGTGTTCCCGACCTGCAGCTCTCCTTG  
71▶ R A I G L L S Q R P L Q A D T Q A A G R R E E  
281 ACATCGGCGGAAGAACATACAAGCAGCTGGACGTCCAGAGGACGTTGAAGGGAGAATGCTGGCGGAAGAG  
TGTAGCCGCTTCTTGATGTTTCGTCGACCTGCAGGTCTCCTGCAACTTCCCTCTTACGACCGCTTCTC  
94▶ H I G G R T Y K Q L D V Q R T L K G E C W R K S  
351 CACACAACAGACATCGGCACGCCAGCAGGCCATCCACCAGAGGAACGACTCGGAGTTTGGCCTGGAGGTG  
GTGTGTGTCTGTAGCCGTGCGGTGCTCCGGTAGGTGGTCTCCTTGCTGAGCCTCAAACCGGACCTCCAC  
117▶ T Q Q T S A R Q Q A I H Q R N D S E F G L E V  
421 AATTTCATAAAGGACAATAGCCGAGCCCTTATTCAAGAATGGGAATGACTGTTATAAGCAAATCAGAG  
TTAAAGTATTTCCCTGTTATCGGCTCGGGAATAAGTTTCTTACCCTTACTGACAATATTTCTGTTTAGTGTC  
141▶ N F I K D N S R A L I Q R M G M T V I K Q I T  
491 ATGACCTATTTGTATGGAATGTTCTGAATCGCGAAGAAGTAAACATCATTTGCTGCGAGAAGGTGGAGCA  
TACTGGATAAATACCTTACAAGACTTAGCGCTTCTTCATTTGTAGTAAACGACGCTCTTCCACCTCGT  
164▶ D D L F V W N V L N R E E V N I I C C E K V E Q  
561 GGATGCTGCTAGAGGGATCATTCACATGATTTTGAAAAAGGGTTGAGAGTCTGTAACTCTTTCTTAA  
CCTACGACGATCTCCCTAGTAAGTGTACTAAACCTTTTCCCAAGTCTCAGGACATTGGAGAAAGAATTT  
187▶ D A A R G I I H M I L K K G S E S C N L F L K  
631 TCCCTTAAGGAGTGGAACCTATCCTCTATTTTCAGGACTTGAATGGACAAAGTTTGTAGGAGACACAGAATT  
AGGGAATTCCTCACCTTGATAGGAGATAAAGTCCTGAACCTTACCTGTTTCAAACTCCTCTGTGTCTTAA  
211▶ S L K E W N Y P L F Q D L N G Q S F E E T Q N  
701 GGGTCTTCTTTAACATCACCTCTTCTCTAATAGGTCTTTTTCATCAGACATCAGAAGGAGACTTGGACGA  
CCCAGAAGAAATGTAGTGGAGAAGAGATTATCCAGAAAAAGTAGTCTGTAGTCTTCTCTGAACCTGCT  
234▶ W V F F N I T S S L I G L F H Q T S E G D L D D  
771 TTTGGCTCAGGATTTAAAGGACTTGTACCATACCCCATCTTTTCTGAACCTTTATCCCTTGGTGAAGAT  
AAACCGAGTCTTAAATTTCTGAAATGTTATGGGGTAGAAAAAGACTTGAAAATAGGGGAACCACTTCTA  
257▶ L A Q D L K D L Y H T P S F L N F Y P L G E D

FIG. 2A

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841 ATTGACATTATTTTAACTTGAAAAGCACCTTCACAGAACCTGTCTGTGGAGGAAGGACCAACACCATC  
TAACTGTAATAAAAAATTGAACTTTTCTGGAAGTGTCTTGGACAGGACACCTCCTTCTGGTTGTGGTAG  
281▶ I D I I F N L K S T F T E P V L W R K D Q H H

911 ACCGCGTGGAGCAGCTGACCCTGAATGGCCTCCTGCAGGCTCTTCAGAGCCCCTGCATCATTGAAGGGGA  
TGGCGCACCTCGTCGACTGGGACTTACCGGAGGACGTCCGAGAAAGTCTCGGGGACGTAGTAACCTTCCCT  
304▶ H R V E Q L T L N G L L Q A L Q S P C I I E G E

981 ATCTGGCAAAGGCAAGTCCACTCTGCTGCAGCGAATTGCCATGCTCTGGGGCTCCGGAAAGTGCAAGGCT  
TAGACCGTTTTCCGTTACAGGTGAGACGACGTTCGCTTAACGGTACGAGACCCCGAGGCCTTTCACGTTCCGA  
327▶ S G K G K S T L L Q R I A M L W G S G K C K A

1051 CTGACCAAGTTCAAATTCGTCTTCTTCTCCGTCTCAGCAGGGCCCAGGGTGGACTTTTTGAAACCCCTCT  
GACTGGTTCAAGTTTAAGCAGAAGAAGGAGGCAGAGTCTCCCGGGTCCACCTGAAAACTTTGGGAGA  
351▶ L T K F K F V F F L R L S R A Q G G L F E T L

1121 GTGATCAACTCTGGATATACCTGGCACAATCAGGAAGCAGACATTTCATGGCCATGCTGCTGAAGCTGGC  
CACTAGTTGAGGACCTATATGGACCGTGTAGTCTTCTGTAAGTACCGGTACGACGACTTCGACGC  
374▶ C D Q L L D I P G T I R K Q T F M A M L L K L R

1191 GCAGAGGGTTCTTTTCTTCTTGATGGCTACAATGAATTCAAGCCCCAGAACTGCCAGAAATCGAAGCC  
CGTCTCCCAAGAAAAGGAAGAACTACCGATGTTACTTAAGTTTCGGGGTCTTGACGGGTCTTTAGCTTCGG  
397▶ Q R V L F L L D G Y N E F K P Q N C P E I E A

1261 CTGATAAAGGAAAACCACCGCTTCAAGAACATGGTCAATCGTCACCACTACCACTGAGTGCCTGAGGCACA  
GACTATTTCTTTTTGGTGGCGAAGTTCTTGTACCACTAGCAGTGGTGTATGGTCACTACCGACTCCCGTGT  
421▶ L I K E N H R F K N M V I V T T T T E C L R H

1331 TACGGCAGTTTGGTGGCCCTGACTGCTGAGGTGGGGATATGACAGAAGACAGCGCCCAGGCTCTCATCCG  
ATGCCGTCAAACCACGGGACTGACGACTCCACCCCTATACTGTCTTCTGTCCGGGTCCGAGAGTAGGC  
444▶ I R Q F G A L T A E V G D M T E D S A Q A L I R

1401 AGAAGTGCTGATCAAGGAGCTTGCTGAAGGCTTGTGCTCCAAATTCAGAAATCCAGGTGCTTGAGGAAT  
TCTTCACGACTAGTTCTCGAACGACTTCCGAACAACGAGGTTTAAGTCTTTAGGTCCACGAACTCCTTA  
467▶ E V L I K E L A E G L L L Q I Q K S R C L R N

1471 CTCATGAAGACCCCTCTCTTTGTGGTCACTACTGTGCAATCCAGATGGGTGAAAGTGAGTTCCACTCTC  
GAGTACTTCTGGGGAGAGAAACACCAGTAGTGAACACGTTAGGTCTACCCACTTTCACTCAAGGTGAGAG  
491▶ L M K T P L F V V I T C A I Q M G E S E F H S

1541 ACACACAACAACGCTGTTCCATACCTTCTATGATCTGTTGATACAGAAAAACAAACACAACATTAAGG  
TGTGTGTTTGTGTCGACAAGGTATGGAAGATACTAGACAACCTATGTCTTTTGTGTTGTGTTGTAATTC  
514▶ H T Q T T L F H T F Y D L L I Q K N K H K H K G

1611 TGTGGCTGCAAGTGACTTCATTCCGAGCCTGGACCACTGTGGAGACCTAGCTCTGGAGGGTGTGTTCTCC  
ACACCGACGTTCACTGAAGTAAGCCTCGACCTGGTGACACCTCTGGATCGAGACCTCCACACAAGAGG  
537▶ V A A S D F I R S L D H C G D L A L E G V F S

1681 CACAAGTTTGATTTTGAAGTGCAGGATGTGTCCAGCGTGAATGAGGATGTCTCTGCTGACAAGTGGGCTCC  
GTGTTCAAAGCTTGAAGCTTACAGCTCTACACAGGTTCGCACTTACTCTACAGGACGACTGTGACCCGAGG  
561▶ H K F D F E L Q D V S S V N E D V L L T T G L

FIG. 2B

1751 TCTGTAAATATACAGCTCAAAGGTTCAAGCCAAAGTATAAATTCTTTTCAAGTCATTCCAGGAGTACAC  
AGACATTATATATGTCGAGTTTCCAAGTTTCGGTTTCATATTTAAGAAAGTGTTCAGTAAGGTCCTCATGTG  
584▶ L C K Y T A Q R F K P K Y K F F H K S F Q E Y T  
-----

1821 AGCAGGACGAAGACTCAGCAGTTTATTGACGTCTCATGAGCCAGAGGAGGTGACCAAGGGGAATGGTTAC  
TCGTCTCTGCTTCTGAGTCGTCAAATACTGCAGAGTACTCGGTCTCCTCCACTGGTTCCCTTACCAATG  
607▶ A G R R L S S L L T S H E P E E V T K G N G Y  
-----

1891 TTGCAGAAAATGGTTTCCATTTTCGGACATTACATCCACTTATAGCAGCCTGCTCCGGTACACCTGTGGGT  
AACGTCTTTTACCAAAGGTAAAGCCTGTAATGTAGGTGAATATCGTTCGGACGAGGCCATGTGGACACCCA  
631▶ L Q K M V S I S D I T S T Y S S L L R Y T C G  
-----

1961 CATCTGTGGAAGCCACCAGGGCTGTTATGAAGCACCTCGCAGCAGTGTATCAACACGGCTGCCTTCTCGG  
GTAGACACCTTCGGTGGTCCCGACAATACTTCGTGGAGCGTCGTACATAGTTGTGCCGACGGAAGAGCC  
654▶ S S V E A T R A V M K H L A A V Y Q H G C L L G  
-----

2031 ACTTTCCATCGCCAAGAGGCCTCTCTCGGAGACAGGAATCTTTGCAAAGTGTGAAAAACACCACTGAGCAA  
TGAAAGGTAGCGGTCTCTCCGAGAGACCTCTGTCTTTAGAAACGTTTCACACTTTTGTGGTGACTCGTT  
677▶ L S I A K R P L W R Q E S L Q S V K N T T E Q  
-----

2101 GAAATTCGTAAAGCCATAAACATCAATTCCTTTGTAGAGTGTGGCATCCATTTATATCAAGAGAGTACAT  
CTTTAAGACTTTTCGGTATTTGTAGTTAAGGAAACATCTCACACCGTAGGTAAATATAGTTCTCTCATGTA  
701▶ E I L K A I N I N S F V E C G I H L Y Q E S T  
-----

2171 CCAAATCAGCCCTGAGCCAAGAATTTGAAGCTTTCTTTTCAAGGTAAAAGCTTATATATCAACTCAGGGAA  
GGTTTAGTCGGGACTCGGTCTTAACTTCGAAAGAAAGTTCATTTTCGAATATATAGTTGAGTCCCTT  
724▶ S K S A L S Q E F E A F F Q G K S L Y I N S G N  
-----

2241 CATCCCCGATTACTTATTTGACTTCTTTGAACATTTGCCCAATTGTGCAAGTGCCCTGGACTTCATTAAA  
GTAGGGGCTAATGAATAAACTGAAGAACTTGTAAACGGGTAAACACGTTACGGGACCTGAAGTAATTT  
747▶ I P D Y L F D F F E H L P N C A S A L D F I K  
-----

2311 CTGGACTTTTATGGGGGAGCTATGGCTTCATGGGAAAAGGCTGCAGAAGACACAGGTGGAATCCACATGG  
GACCTGAAAATACCCCTCGATACCGAAGTACCCTTTTCCGACGCTCTCTGTGTCCACCTTAGGTGTACC  
771▶ L D F Y G G A M A S W E K A A E D T G G I H M  
-----

2381 AAGAGGCCCCAGAAACCTACATTTCCAGCAGGGCTGTATCTTTGTTCTTCAACTGGAAGCAGGAATTCAG  
TTCTCCGGGGTCTTTGGATGTAAGGGTCGTCCCGACATAGAAACAAGAAGTTGACCTTCGTCTCTTAAGTC  
794▶ E E A P E T Y I P S R A V S L F F N W K Q E F R  
-----

2451 GACTCTGGAGGTCACACTCCGGGATTTTCAGCAAGTTGAATAAGCAAGATATCAGATATCTGGGGAAAATA  
CTGAGACCTCCAGTGTGAGGCCCTAAAGTCGTTCAACTTATTCGTTCTATAGTCTATAGACCCCTTTTAT  
817▶ T L E V T L R D F S K L N K Q D I R Y L G K I  
-----

2521 TTCAGCTCTGCCACAAGCCTCAGGCTGCAAATAAAGAGATGTGCTGGTGTGGCTGGAAGCCTCAGTTTGG  
AAGTCGAGACGGTGTTCGGAGTCCGACGTTTATTTCTCTACACGACCACACCGACCTTCGGAGTCAAACC  
841▶ F S S A T S L R L Q I K R C A G V A G S L S L  
-----

2591 TCCTCAGCACCTGTAAGAACATTTATCTCTCATGGTGGAAAGCCAGTCCCTCACCATAGAAGATGAGAG  
AGGAGTCGTGGACATTTCTGTAAATAAGAGAGTACCACCTTCGGTCAGGGGAGTGGTATCTTCTACTCTC  
864▶ V L S T C K N I Y S L M V E A S P L T I E D E R  
-----

FIG. 2C

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1561 ATGATCAGATCTCTTACGAAACCTGAAACCTTGAATATGATGACCTACAGATCAACGGCTGCGGGT  
TGTGTAGTGTAGACATTTGTTTGAATTTGGAACTCATAGTACTGGATGCTTATGTTGCGCAAGGCGCA  
387 H I T S V T N L K T L S I H D L O N Q R L P G

1731 ATCTGACTGACAGCTTGGGTAACTTGAAGAACCTTCAAGCTCATATGGATACATTAAGATGAATG  
CCAGACTGACTGTGGAACCCATTTGAACCTTCTTGAATGTTCGAGTATACCTATTGTATTCTACTTAC  
911 G L T D S L G N L K N L T K L I M D N I K M N

1801 AAGAGATGCTATATAAACTAGCTGAGGCTTGAANAACCTGAGAAGATGTGTTTATTTCAATTTGACCCA  
TCTTCTACGATATTTGATCGACTTCCGACTTTTGGACTTCTTCTACACAAATAAAGTAACTGGGT  
934 E E D A I K L A E G L K N L K K M C L F H L T H

1871 TTTGTCTGACATTGGAGAGGGATGGATTACATAGTCAAGTCTCTGTCAAGTGAACCTGTGACCTTGAA  
GAACAGACTGTAACTCTCCCTTACCTAATGTATCAGTTCAGAGACAGTTCACTTGGGACACTGGAACCT  
957 L S D I G E G M D Y I V K S L S S E P C D L E

1941 GAAATTCATTAGTCTCTGCTGCTTGTCTGCAATGCAGTGAANAATCTAGCTCAGAATCTTCACAATT  
CTTTAAGTTAATCAGAGGACGACGAACAGACGTTTACGTCACTTTTAGGATCGAGTCTTAGAAGTGTAA  
981 E I O L V S C C L S A N A V K I L A O N L H N

3011 TGGTCAAACTGAGCATTTCTTGATTTATCAGAAAATTACCTGGAAAAAGATGGAAATGAAGCTCTTCATGA  
ACCAGTTTGACTCGTAAGAACTAAATAGTCTTTAATGGACCTTTTCTACCTTTACTTCGAGAAGTACT  
1004 L V K L S I L D L S E N Y L E K D G N E A L H E

3081 ACTGATCGACAGGATGAACGCTGCTAGAACAGCTCACCGCACTGATGCTGCCCTGGGGCTGTGACGTGCAA  
TGACTAGCTGTCTACTTGCACGATCTTGTGAGTGGCGTGACTACGACGGGACCCCGACACTGCCAGTT  
1027 L I D R M N V L E Q L T A L M L P W G C D V Q

3151 TCCAGCCTGAGCAGCCTGTTGAAACATTTGAGGAGGTCCCACAACTCGTCAAGCTTGGGTGAAAACT  
CCGTCCGACTCGTCCGACAACCTTTGTAACCTCTCCAGGGTGTGAGCAGTTCGAACCCAACTTTTGA  
1051 G S L S S L L K H L E E V P Q L V K L G L K N

3221 TTAGACTCAGATACAGAGATTAGAATTTTGGGTGCAATTTTGGAAAGAACCTCTGAAAACTTCCA  
CCTCTGAGTGTCTATGTCTTAATCTTAAATCCACGTAAAAAACCTTTCTTGGGAGACTTTTGAAGGT  
1074 W R L T D T E I R I L G A F F G K N P L K N F Q

3291 TCAAGTTGAATTTGGCGGAAATCGTGTGAGCAGTGTGATGGATGGCTTGCCTTCATGGGTGTATTGAGAAT  
CGTCAACTTAAACCGCCCTTTAGCACACTCGTCACTACCTACCGAACCGAAGTACCCACATAAACTCTTA  
1097 Q L N L A G N R V S S D G W L A F M G V F E N

3361 TTTAAGCAATTAGTGTTTTTGACTTTAGTACTAAAGAATTTCTACCTGATCCAGCATTAGTCAGAAAAC  
GAATTCGTTAATCAGAAAACTGAATCATGATTTCTTAAAGATGGACTAGGTGTAATCACTCTTTG  
1121 L K O L V F F D F S T K E F L P D P A L V R K

3431 TTAGCCAGTGTATCCAGTTAACTTTCTGCAAGAAGCTAGGCTTGTGGGTGGCAATTTGATGATGA  
AATCGGTTCAATAGGTTCAATTGAAAAGACGTTCTTCGATCCGAACCCACCGTTAACTACTACT  
1144 L S O V L S K L T F L Q E A R L V G W Q F D D D

3501 TGATCTCAGTGTATTACAGATGAGAAAGCTCAGATGATTTGCCCATGGGTATATAAACTACTTCTTAC  
ACTAGAGTCACAATAATGTCTACTCTTTCGAGTCTACTAAACGGGTACCCAATATTTTGATGAAGGAATG  
1167 D L S V I T D E K A Q M I C P W V I K L L P Y

FIG. 2D

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3571 ACAGTGGCAGCATCAGAACTGGAATTCAGATCTCTTGCCTCCTAG  
TGTACCGTCGTCGTAGTCTTGACCTTAAGTCTAGAGAACGGAGGATC  
1191▶ T V A A S E L E F R S L A S

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FIG. 2E



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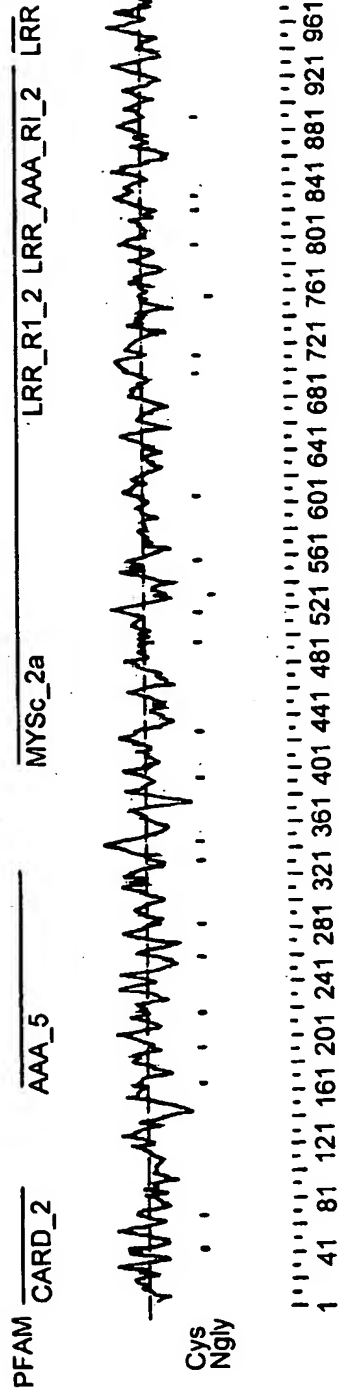
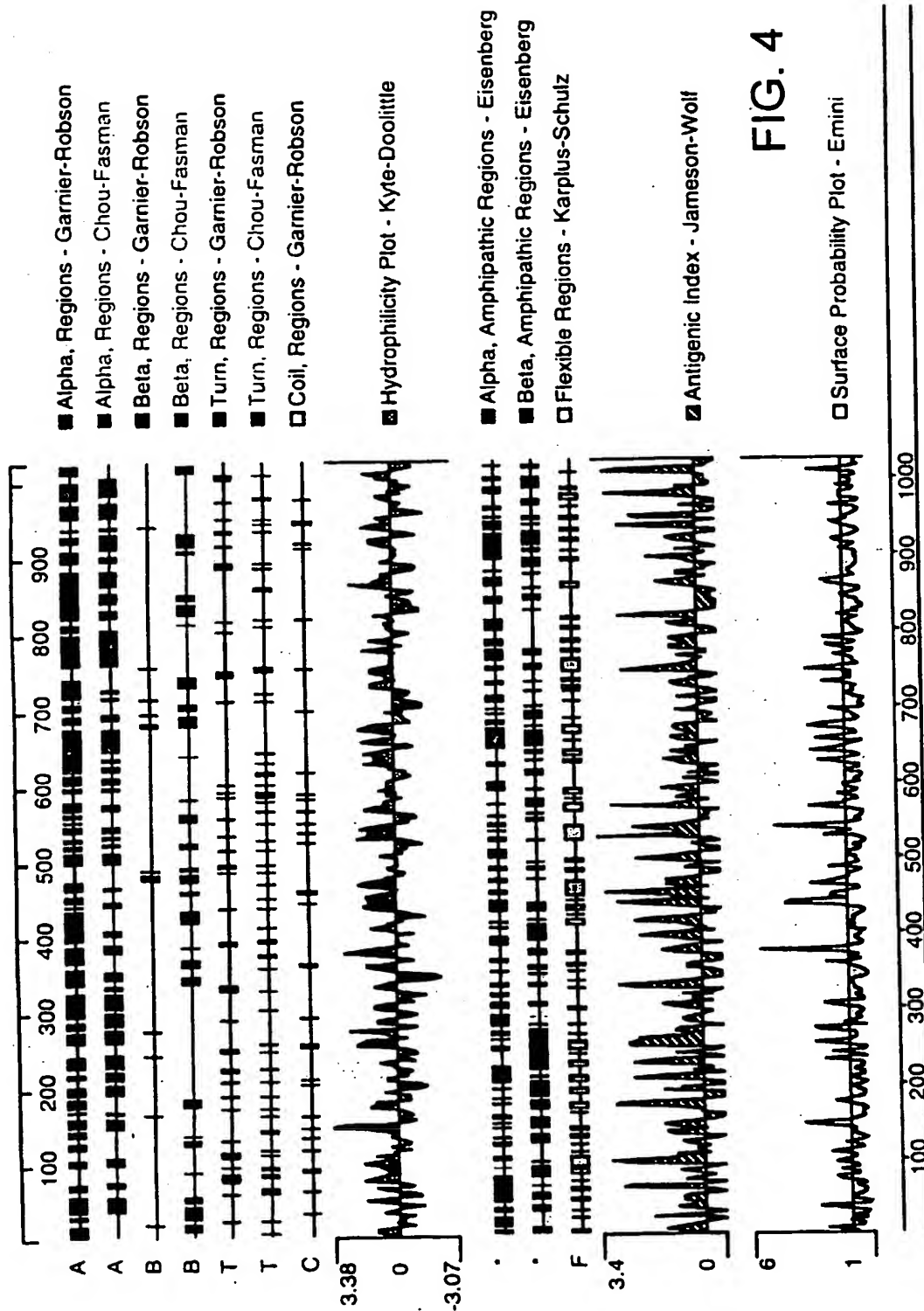


FIG. 3

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**CARD:** domain 1 of 1, from 2 to 88: score 16.0, E = 0.0065

```
*->aeddrlllrknrlgelgtlsglldhLlleknvLteeeeeEkikaknt
+ ++ ++ + +g +++++ d+L nvL+eee+ i +
2 --NFIKDNSRALIQRMGMTVIKQITDDLFVWVNLNREEVNIICCEKV 46

CARD12

trrdkareLiDsvqkGnqAqfiFlqaLretdqelladllde<-*
++ d ar i .+++kkG++ ++F1 +L+e ++ l +d1 +
47 EQ-DAARGIIHMLKKGSESCNLFKLKSLKEWNYPLFQDLNGQS 88

CARD12
```

**FIG. 5A**

LRR: domain 1 of 4, from 764 to 791: score 0.6, E = 8.2e+02	
*->nLeeLdLsnN.Lt.....slppgIfsnLp<-*	
nL++L ++n + +++ +l +g ++nL+	
NLTKLIMDNIKMNNeedaiKLAEG-LKNLK	791
CARD12 764	FIG. 5B
LRR: domain 2 of 4, from 821 to 848: score 0.3, E = 9.3e+02	
*->nLeeLdLsnN.Lt.....slppgIfsnLp<-*	
Lee+ L ++ L+ + ++ ++nL	
DLEEIQLVSCcLSanavKILAQNHLNLV	848
CARD12 821	FIG. 5C

**FIG. 5D**

<b>LRR:</b> domain 3 of 4, from 849 to 872: score 11.2, E = 23	
*->nLeeLdLsnN.LtSlppgIfsnLp<-*	
+L LdLs N L++ +++++ L	
CARD12 849 KLSILDLSenYLEKDGNEALHELI	872
	<b>F</b>
<b>LRR:</b> domain 4 of 4, from 938 to 965: score 4.2, E = 2.5e+02	
*->nLeeLdLsnN.Lt.....slppgIfsnLp<-*	
n + L+L +N+ +++ + +f+nL+	
CARD12 938 NFQQLNLAGNrVSSdgwLAFMG-VFENLK	965

# FIG. 5E

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		E . L . L . . . . . L . S . . . . . E G E . G . G K . . . L L . . I A . L W . S G																																Consensus #1								
		E Q L V L N G V L G A L N S V C I V E G E A G S G K S V L L O K I A F L W G S G																																Majority								
		10								20								30								40																
1		E	Q	L	T	L	N	G	L	L	Q	A	L	Q	S	P	C	I	I	E	G	E	S	G	K	G	K	S	T	L	L	Q	R	I	A	M	L	W	G	S	G	CARD12-C
1		E	P	L	V	L	P	E	V	F	G	N	L	N	S	V	M	C	V	E	G	E	A	G	S	G	K	T	V	L	L	K	K	I	A	F	L	W	A	S	G	NAIP-C
		. C . . L . . F . . V F . L . L S . . R . . . G L . . . . C D Q L L . . . G . .																																Consensus #1								
		K C K A L T R F O L V F F L S L S S T R A D G G L A S I L C D Q L L D I E G S V																																Majority								
		50								60								70								80																
41		K	C	K	A	L	T	K	F	K	F	V	F	F	L	R	L	S	-	-	R	A	Q	G	G	L	F	E	T	L	C	D	Q	L	L	D	I	P	G	T	I	CARD12-C
41		C	C	P	L	L	N	R	F	Q	L	V	E	Y	L	S	L	S	S	T	R	P	D	E	G	L	A	S	I	I	C	D	Q	L	L	E	K	E	G	S	V	NAIP-C
		. . . . . L . . . V L F L L D . Y . E . . . . . C . . . . . I . . L																																Consensus #1								
		T E Q T F R A I L L Q L K N Q V L F L L D G Y N E I K P Q N C S I P Q V I G A L																																Majority								
		90								100								110								120																
79		R	K	Q	T	F	M	A	M	L	L	K	L	R	Q	R	V	L	F	L	L	D	G	Y	N	E	F	K	P	Q	N	C	P	E	-	-	-	I	E	A	L	CARD12-C
81		T	E	M	C	M	R	N	I	I	Q	Q	L	K	N	Q	V	L	F	L	L	D	D	Y	K	E	I	-	-	-	-	C	S	I	P	Q	V	I	G	K	L	NAIP-C
		I . . N H . . . . . T . . . R . I R . . . . . E . . . . .																																Consensus #1								
		I Q E N H L S K T C V L V A V T T E R A R D I R Q F G A L I A E V G A F T E D S																																Majority								
		130								140								150								160																
116		I	K	E	N	H	R	F	K	N	M	V	I	V	T	T	T	E	C	L	R	H	I	R	Q	F	G	A	L	T	A	E	V	G	D	M	T	E	D	S	CARD12-C	
117		I	Q	K	N	H	L	S	R	T	C	L	L	I	A	V	R	T	N	R	A	R	D	I	R	Y	L	E	T	I	L	E	I	Q	A	F	P	F	Y	N	NAIP-C	
		. . . . . R . . . . . K . . . L . . . . K T P L F V .																																Consensus #1								
		A V A L L R E V L I K E L A E L R G L L V Q I G K S Q S L Q N L O K T P L F V A																																Majority								
		170								180								190								200																
156		A	Q	A	L	I	R	E	V	L	I	K	E	L	A	E	-	-	G	L	L	L	Q	I	Q	K	S	R	C	L	R	N	L	M	K	T	P	L	F	V	V	CARD12-C
157		T	V	C	I	L	R	K	L	F	S	H	N	M	T	R	L	R	K	F	M	V	Y	F	G	K	N	Q	S	L	Q	K	I	Q	K	T	P	L	F	V	A	NAIP-C
		. . C A . . . . . F . . . . . F . . . . . L . . . N K . . . . .																																Consensus #1								
		A I C A I Q W G E S E F D S S F T D V A V F K S F Y D L L I L K N K H K H K G V																																Majority								
		210								220								230								240																
194		I	T	C	A	I	Q	M	G	E	S	E	F	H	S	H	-	T	Q	T	T	L	F	H	T	F	Y	D	L	L	I	Q	K	N	K	H	K	H	K	G	V	CARD12-C
197		A	I	C	A	-	H	W	F	Q	Y	P	E	D	P	S	F	D	D	V	A	V	E	K	S	Y	M	E	R	L	S	L	R	N	K	-	-	-	-	-	NAIP-C	
		A . . . . . C G . L A L . G . F S . . F . F . . . D . . . . V . E																																Consensus #1								
		A A A D I L K A T V S S C G D L A L E G V F S H K F D F E L D D V A E A G V D E																																Majority								
		250								260								270								280																
233		A	A	S	D	F	I	R	S	-	L	D	H	C	G	D	L	A	L	E	G	V	F	S	H	K	F	D	F	E	L	Q	D	V	S	-	-	S	V	N	E	CARD12-C
230		A	T	A	E	I	L	K	A	T	V	S	S	C	G	E	L	A	L	K	G	F	F	S	C	C	F	E	E	N	D	D	D	L	A	E	A	G	V	D	E	NAIP-C
		D . . L T . . L . . K . T A Q R . . P . Y . F . . . . F Q E . . A G . R L . . L																																Consensus #1								
		D V L L T T G L L S K F T A Q R L K P K Y K F L S K A F Q E F L A G R R L I S L																																Majority								
		290								300								310								320																
270		D	V	L	L	T	T	G	L	L	S	K	F	T	A	Q	R	L	K	P	K	Y	K	F	F	H	K	S	F	Q	E	Y	T	A	G	R	R	L	S	S	L	CARD12-C
270		D	E	D	L	T	M	C	L	M	S	K	F	T	A	Q	R	L	R	E	F	Y	R	E	L	S	P	A	F	Q	E	F	L	A	G	M	R	L	I	E	L	NAIP-C

FIG. 6A

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		L . S . . . . E . . . . . G . . . . L . . . . S . . . . . S . Y . . . L . Y . . . . .	Consensus #1
		L T S D E Q E E V T L G L G H L Q Q I V S I S D I V S A Y S S L L N Y V S G L S	Majority
		330 340 350 360	
310	L T S H E P E E V T K G N G Y L Q K M V S I S D I T S T Y S S L L R Y T C G - S	CARD12-C	
310	L D S D R Q E H Q D L G L Y H L K Q I N S P M M T V S A Y N N F L N Y V S S L P	NAIP-C	
		S . . A . . . . . H L . . . . . L . . . . S . . . . . L . . Q . . . . .	Consensus #1
		S V E A G R A V V S H L A A V V D N K G S L L G L S I A D D Y L K H Q E S I S L	Majority
		370 380 390 400	
349	S V E A T R A V M K H L A A V Y Q H - G C L L G L S I A K R P L W R Q E S - -	CARD12-C	
350	S T K A G P K I V S H L L H L V D N K E S L E N I S E N D D Y L K H Q P E I S L	NAIP-C	
		. . . . L . . . . . Q . . . . . V . . . . . Y Q . . T . .	Consensus #1
		Q M Q L L Q G V K N I T E Q A I L S A V S I N L L V L A G I T A Y Q S S T V A A	Majority
		410 420 430 440	
385	- - - - L Q S V K N T T E Q E I L K A I N I N S F V E C G I H L Y Q E S T S K S	CARD12-C	
390	Q M Q L L R G L W Q I C P Q A Y F S M V S E H L L V L A L K T A Y Q S N T V A A	NAIP-C	
		. . . . . F . Q G . . L . . . . . N . . . Y . . . F F . H . P . . . S . L	Consensus #1
		A L S Q V L E A F L Q G K S L T L G A G N L P D Y L F D F F D H L P E S A S A L	Majority
		450 460 470 480	
421	A L S Q E F E A F F Q G K S L Y I N S G N I P D Y L F D F F E H L P N C A S A L	CARD12-C	
430	C S P F V L Q - F L Q G R T L T L G A L N L - Q Y - - - F E D H - P E S L S L L	NAIP-C	
		. . I . . . . . G . . . . . P . . . . . Y . .	Consensus #1
		D S I K L S I R G G A T A S R A K A A V L T G G I D K S E A P T I D E T Y I P A	Majority
		490 500 510 520	
461	D F I K L D F Y G G A M A S W E K A A E D T G G I H M E E A P - - - E T Y I P S	CARD12-C	
464	R S I H F S I R G N K T S P R A H F S V L E T C F D K S Q V E T I D Q D Y - - A	NAIP-C	
		. A . . . . . W . . . . . E . . . . . L . . . . .	Consensus #1
		S A V S L F N E W E Q E L A T L E V T V K S F S D L N K Q A I T D L G T G F S S	Majority
		530 540 550 560	
498	R A V S L F F N W K Q E F R T L E V T L R D F S K L N K Q D I T Y L G K I F S S	CARD12-C	
502	S A F E P M N E W E R N L A E K E D N V K S Y M D M Q R R A S P D L S T G Y W K	NAIP-C	
		. . . . . I . . C . . . . . L . V . . . . .	Consensus #1
		A S S L Q L Q I K R C A G V A G S L S L V L S T C K N I Y S L E V D A S D L T V	Majority
		570 580 590 600	
538	A T S L R L Q I K R C A G V A G S L S L V L S T C K N I Y S L M V E A S P L T I	CARD12-C	
542	L S P K Q Y K I - P C - - - - - - - - - - - - - - - L E V D V N D I D V	NAIP-C	
		. . . . . S . . . . . Q R . . . . L . . S . . . . .	Consensus #1
		V G E D H L T I V T N L T V L S I H D L A S Q R L E G G L T D S L G N L K G L I	Majority
		610 620 630 640	
578	E D E R H I T S V T N L K T L S I H D L Q N Q R L P G G L T D S L G N L K N L T	CARD12-C	
562	V G Q D M L E I L - - M T V F S - - - - - A S Q R I E L H L N H S - - - - - R G F I	NAIP-C	

FIG. 6B

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		. . . I . . . . . K . . . . . C . . . . . L S . . . . .																				Consensus #1	
		E L I R D A L E L S E A S A I K L A E G L K N L K K M C L I S L L E L S A A G E																				Majority	
		650					660					670					680						
618	K L I	M D N I K M N E E D A I	K	L A E G L K N L K K M	C	L F H L T H L S	D I G E														CARD12-C		
592	E S I	R P A L E L S K A S V T	K	- - - - -											C	S I S K L E L S	A A E Q					NAIP-C	
		. . . . . S L . S . . . . . L E . . . . . Q . . . . N																				Consensus #1	
		G L L L I V K S L S S E P C D L E E I Q L V S C C L V A G A V Q I L A Q I L H N																				Majority	
		690					700					710					720						
658	G M D Y I V K	S L S	S	E P C D	L E E	I Q L V S C C L S A N A V K I L A	Q N L H N														CARD12-C		
621	E L L L T L P	S L E S	- - - -			L E	- - - - - V S G T I Q S Q D											Q I F P N					NAIP-C
		L . K . . . . L . L . E . . . . . G N . . . . . I . . . . . L .																				Consensus #1	
		L V K L S I L D L S E L S V D L D G N I A V H S V I P D E F N V L E Q L T A L L																				Majority	
		730					740					750					760						
698	L V K L S I L	D L S	E N Y L E K D	G N E A L H E L I	- D R M N V L E Q L T A L M															CARD12-C			
648	L D K F - -	L C L K E	L S V D L E G N	I N V F S V I	P E E F P N F H H M E K L L															NAIP-C			
		. . . . . S . L . . . . L . . . . L . L . . . . D . E . .																				Consensus #1	
		L O I G A D V D G S L S S L V A S L E E V I S L V I L G L E G Q Q L T D T E I S																				Majority	
		770					780					790					800						
737	L P W G C D V	Q G S L S	S L L K H L	E E V P Q L V K L G L K N W R L T D T E I R																CARD12-C			
686	I Q I S A E Y	D P - -	S K L V A S L P N F I S L K I L N L E G Q Q F P D E E T S																	NAIP-C			
		. . . . . A . . . . . L . N . . . . L . L . . . . . G . . . . . K																				Consensus #1	
		I L G A F I G L G S L S N L E E L I L A G G D V S S D G W L A F M G V F E V A K																				Majority	
		810					820					830					840						
777	I L G A F F G K N P L K N F Q Q L N L A G N R V S S D G W L A F M G V F E N L K															CARD12-C							
724	E K F A Y I - L G S L S N L E E L I L P T G D - - - - - G I Y R V A K															NAIP-C							
		. . . . . L L V F F D F S T K E F L P D P A L V Q Q L S Q V L S V L S F L Q T A R L V G W																				Consensus #1	
		. . . . . L L V F F D F S T K E F L P D P A L V Q Q L S Q V L S V L S F L Q T A R L V G W																				Majority	
		850					860					870					880						
817	Q L V F F D F S T K E F L P D P A L V R K L S Q V L S K L T F L Q E A R L V G W															CARD12-C							
753	L I I - - - - - Q Q C Q Q L - H C L R V L S E F F K T - - - - -															NAIP-C							
		. . . . . D D . . . . . I . . . . .																				Consensus #1	
		Q L D D D S V V V I T G A F K L V T G																				Majority	
		890																					
857	Q F D D D D L S V I T G A F K L V T A															CARD12-C							
773	- L N D D S V V E I - - - - - G															NAIP-C							

Consensus 'Consensus #1': When all match the residue of CARD12-C show the residue of CARD12-C, otherwise show '.'.

Decoration 'Decoration #1': Box residues that match the consensus named 'Consensus #1' exactly.

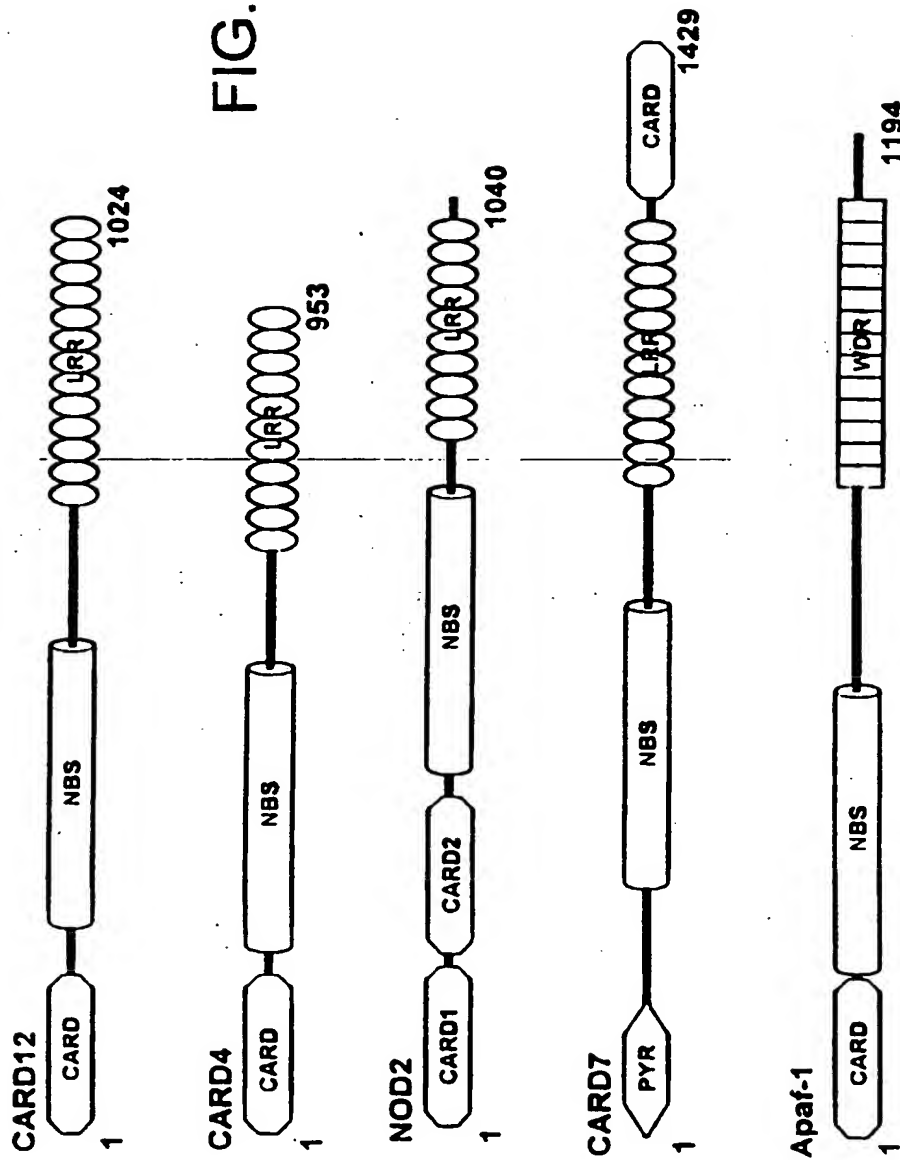
FIG. 6C

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CARD12 **N** **N** **F** **K** **D** **N** **S** **R** **A** **L** **I** **Q** **R** **M** **G** **M** **T** **V** **I** **K** **Q** **T** **D** **D** **L** **F** **V** **W** **N** **V** **L** **N** **R** **E** **E** **V** **N** **I** **J** **C**  
 CARD4 **E** **S** **H** **P** **H** **I** **Q** **L** **I** **K** **S** **N** **R** **E** **L** **I** **V** **I** **H** **I** **R** **N** **T** **Q** **C** **L** **V** **D** **N** **I** **L** **K** **N** **D** **Y** **F** **S** **A** **I** **D** **A** **E** **I** **N** **C** **A**  
 CARD7 **L** **D** **A** **P** **Q** **L** **I** **H** **E** **V** **D** **Q** **Y** **R** **E** **Q** **I** **A** **R** **V** **T** **S** **V** **E** **V** **L** **D** **K** **I** **L** **H** **G** **Q** **V** **L** **S** **Q** **E** **Q** **Y** **E** **R** **V** **L** **A**  
 APAFI **N** **D** **A** **K** **A** **R** **N** **C** **L** **I** **Q** **H** **R** **E** **A** **I** **E** **K** **D** **I** **K** **T** **S** **I** **N** **D** **H** **M** **I** **S** **D** **G** **F** **I** **T** **I** **S** **E** **E** **K** **V** **R** **N**  
 CARD12 **E** **K** **V** **E** **Q** **D** **A** **A** **R** **G** **I** **I** **H** **M** **H** **L** **K** **N** **G** **S** **E** **S** **C** **N** **L** **F** **L** **K** **S** **I** **K** **E** **W** **N** **Y** **P** **I** **F** **Q** **D** **L** **N** **G** **Q** **S**  
 CARD4 **C** **P** **T** **O** **P** **D** **K** **V** **R** **K** **I** **L** **D** **I** **V** **Q** **S** **N** **G** **E** **I** **V** **S** **E** **F** **F** **P** **Y** **L** **L** **Q** **L** **A** **D** **A** **Y** **V** **D** **I** **R** **P** **W**  
 CARD7 **E** **N** **T** **R** **P** **S** **Q** **N** **R** **K** **L** **F** **S** **I** **S** **Q** **S** **W** **D** **R** **K** **C** **K** **D** **G** **P** **Y** **Q** **A** **E** **K** **E** **T** **H** **P** **H** **I** **M** **E** **L** **W**  
 APAFI **E** **P** **T** **Q** **Q** **Q** **R** **A** **A** **M** **I** **I** **K** **M** **I** **I** **K** **N** **D** **N** **S** **Y** **V** **S** **F** **Y** **N** **A** **L** **L** **H** **E** **G** **Y** **K** **D** **I** **A** **A** **I** **I** **H** **D**

FIG. 7

FIG. 8





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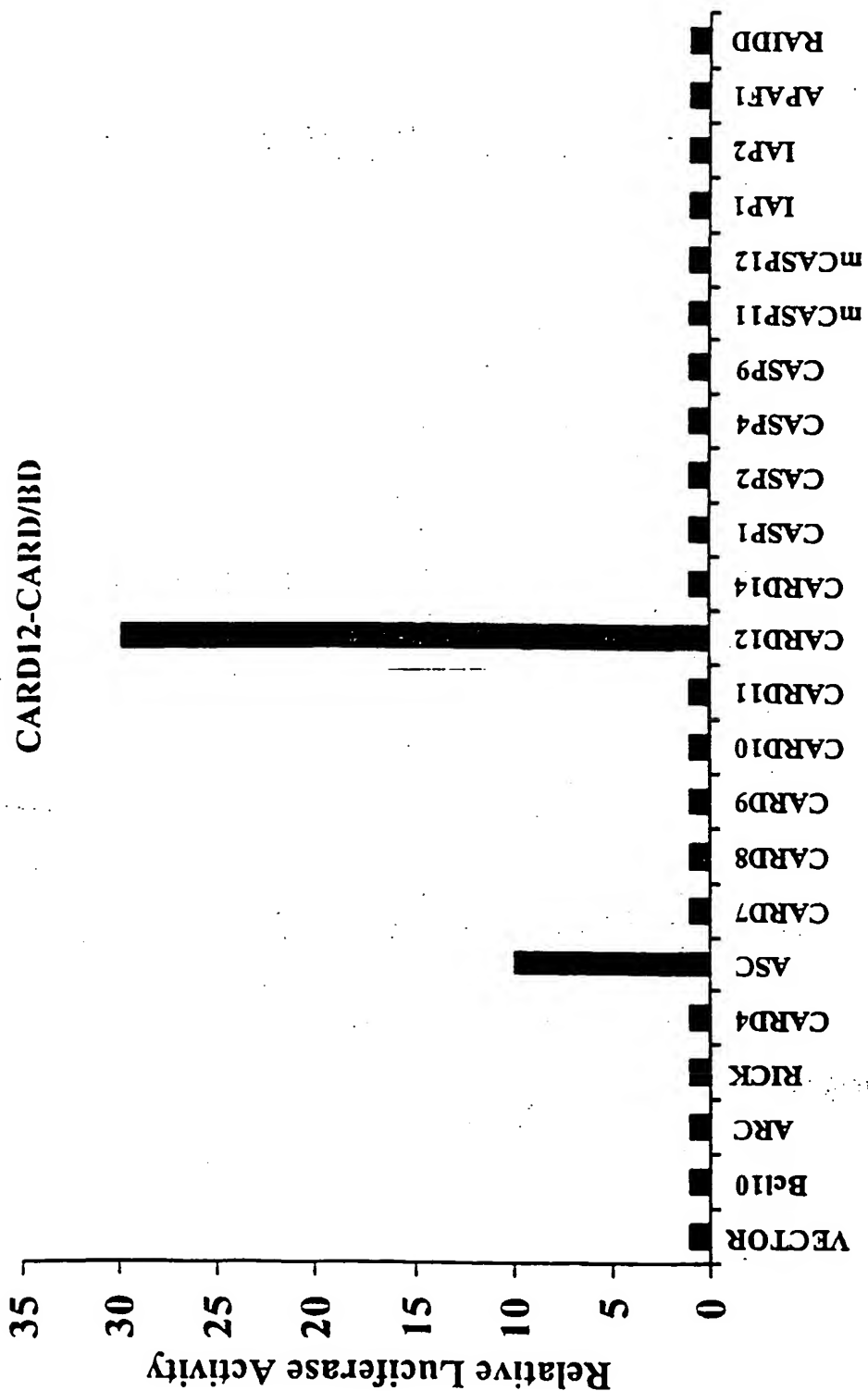


FIG. 6

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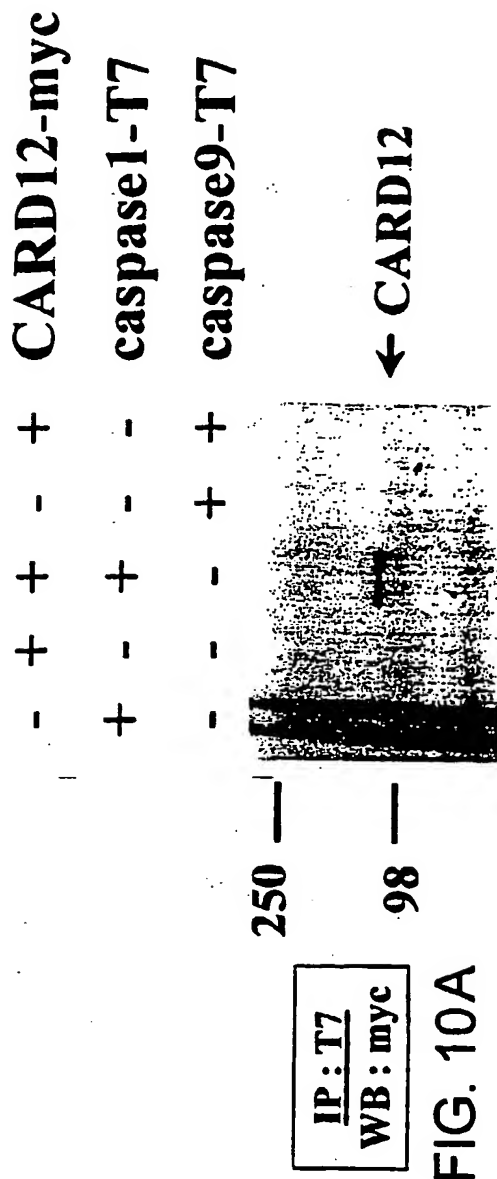


FIG. 10A



FIG. 10B

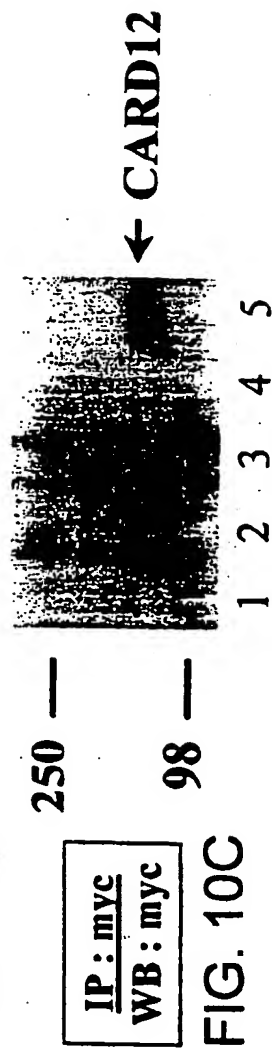


FIG. 10C

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/13009

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07K 14/47, 7/08, 16/18, C12N 1/00, 5/00, 5/06, 15/12, C12P 21/02, C12Q 1/37, 1/68, G01N 33/53, 33/566, 33/68  
US CL : 435/6, 7.8, 23, 69.1, 325, 410, 252.3, 254.1; 436/501; 530/300, 350, 387.9; 536/23.2

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.8, 23, 69.1, 325, 410, 252.3, 254.1; 436/501; 530/300, 350, 387.9; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Genbank, Accession Number AQ309404, ADAMS et al. 'Use of a random human BAC End Sequence Database for Sequence-Ready Map Building.' National Library of Medicine, Bethesda MD, 22 December 1998, (complement of nt 2-552 100% identical to nt 1462-2012 of SEQ ID NO:1), Gene Sequence.	1-6, 16-20, 30- 32
A	HOFMANN. The modular nature of apoptotic signaling proteins. Cell. Mol. Life Sci. July 1999, Vol. 55, pages 1113-1128, see entire document.	1-35
X, P	WO 01/30971 A2 (MILLENNIUM PHARMACEUTICALS, INC.) 03 May 2001(03.05.01), entire document.	1-35
X, P	DAMIANO et al. CLAN, A Novel Human CED-4-like Gene. Genomics, July 2001, Vol. 75, Nos. 1-3, pages 77-83, entire document.	1-35



Further documents are listed in the continuation of Box C.



See patent family annex.

### \* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

24 July 2002 (24.07.2002)

Date of mailing of the international search report

27 AUG 2002

Name and mailing address of the ISA/US

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# INTERNATIONAL SEARCH REPORT

PCT/US02/13009

**Continuation of B. FIELDS SEARCHED Item 3:**

DIALOG File Medline; WEST files USPT, EPAB, JPAB, DWPI; CA files registry, CA search terms: card?, apoptosis, caspase, recruit?,